Vol. X, No. 3 October 1916

THE BIOCHEMICAL JOURNAL

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1916

Price Seven Shillings net.

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XXVIII. THE VISCOSITY AND HYDRATATION OF COLLOIDAL SOLUTIONS.

By EMIL HATSCHEK.

(*Received July 13th, 1916.*)

In a paper published in this *Journal* Miss Chick [1914] has calculated the hydratation factors for a number of proteins from the author's formula for the viscosity of emulsoid sols. By means of this formula the volume of the disperse phase, i.e. the hydrated protein, is obtained from the relative viscosity, and the hydratation or association factor is then found by comparing this volume with the weight of dissolved substance. Miss Chick's results are: (1) that for each protein the association factor is constant over a considerable range of concentration, and (2) that its value lies between 1·57 for crystallised egg albumin and 8·63 for sodium caseinogenate, these figures being the numbers of grams of water associated with each gram of protein in solution.

In a recent paper under the above title Arrhenius [1916] criticises these results and considers them open to grave doubt, chiefly on the ground that the factors are very much larger than those determined (by other methods) in the case of the most highly hydrated salts in solution, such as $MgCl_2 + 12H_2O$ (equivalent to about 2·3 g. of water per g. of salt), which hydrate is known only at $-16{\cdot}8°$, while the viscosity figures for proteins were obtained at a temperature of 25°.

Arrhenius then applies to the viscosity figures treated by Miss Chick his well-known formula:

$$\log \eta = \theta C, \quad \text{.....(1)}$$

in which C however now means the molecular concentration, while θ is a constant and η the relative viscosity. For substances like proteins, whose molecules are extremely large compared with those of water, he considers that C is expressed with sufficient accuracy by the equation

$$C = \frac{100p}{100 - (n+1)p}, \quad \text{.....(2)}$$

in which p is the number of g. of solute in 100 g. of solution, and n the hydratation factor, i.e. the number of g. of solvent associated with each g. of solute, and therefore withdrawn from the free solvent.

The complete expression for the logarithm of the viscosity of a solution, in which the molecular weight of the solute is high compared with that of the solvent, is accordingly:

$$\log \eta = \theta \frac{100p}{100 - (n+1)p}, \quad \text{.....(3)}$$

and as this expression contains only two constants, θ and n, it is obvious that these can be calculated from two sets of experimental data. Solving equation (3) for $(n + 1)$ we obtain:

$$n + 1 = \frac{100}{p} - \frac{100\theta}{\log \eta}, \quad \text{.....(4)}$$

and if we introduce two pairs of coordinated values p_1, η_1, and p_2, η_2:

$$n + 1 = \frac{100}{p_1} - \frac{100\theta}{\log \eta_1} = \frac{100}{p_2} - \frac{100\theta}{\log \eta_2}. \quad \text{.....(5)}$$

By elimination we find the value of θ:

$$\theta = \left(\frac{1}{p_1} - \frac{1}{p_2}\right) : \left(\frac{1}{\log \eta_1} - \frac{1}{\log \eta_2}\right), \quad \text{.....(6)}$$

and by introducing the value thus found into either of the equations (5) the value of n. It is not very clear why Arrhenius suggests that n should be found by trial, as the strict procedure is certainly more expeditious as well as more elegant.

The formula (3), when applied to Miss Chick's viscosity figures, fits with remarkable accuracy, and the values of θ and n are constants over the whole range with slight exceptions. The agreement is least satisfactory for sodium caseinogenate, which shows the highest viscosity for a given weight of protein, and Arrhenius ascribes the latter to dissociation.

In order to get away from the analogy with hydrated salts—the cogency of which the author must confess himself unable to admit—and from dissociation, it seems highly desirable to test Arrhenius's formula by applying it to sols in organic solvents, such as india rubber and nitrocellulose sols. Although dissociation is certainly absent in both cases, these colloids produce, for the same weight of substance, rises in viscosity which are enormous compared even with sodium caseinogenate. They may also be expected to be highly solvated or associated, as the criterion from which Arrhenius anticipates a high degree of hydratation in the protein sols applies even more emphatically: viz. they swell enormously in their respective solvents.

The data to be used below are taken, in the case of rubber, from a paper by Schidrowitz and Goldsbrough [1909], and in that of nitrocellulose from a very complete investigation by Frank Baker [1913]. The first column shows the concentration as stated by the authors, viz. g. per 100 cc. of solution. As Arrhenius's formula contains the concentration by weight, the second column shows p, the *weight* of substance in 100 *g. of solution*. These values are exact for the nitrocellulose sols, as the densities of the sols are given by the author; for the rubber sols the densities are not available and the value of p is therefore approximate only, but in view of the low concentrations the possible error is certainly slight.

From these values of p and the observed viscosities (stated in all cases as relative viscosities, that of the solvent being taken as unity) the constants θ and n have been calculated by solving the equations (5) and (6). This involves the assumption that the method used for calculating the molecular concentration of proteins in water is equally applicable to rubber in benzene, and nitrocellulose in acetone or ether-alcohol—an assumption which is no doubt sufficiently correct, although the molecules of solute are smaller and those of solvent larger, than the molecules of protein and water respectively.

As a first example we may take a typical rubber sol (hard Para in benzene):

	g. in 100 cc.	p	η	$\log \eta$
(1)	0·25	0·281	2·5	0·39794
(2)	0·50	0·562	6·8	0·83251
(3)	1·00	1·124	32·3	1·50920

From these three sets of values we can form three pairs of equations and can thus calculate θ and n in three different ways. We thus obtain the following values:

From (1) and (2)	$\theta = 1{\cdot}354$	$n = 14{\cdot}4$,
From (1) and (3)	$\theta = 1{\cdot}441$	$n = -7{\cdot}5$,
From (2) and (3)	$\theta = 1{\cdot}652$	$n = -21{\cdot}5$.

These values are so divergent that the formula cannot be said to apply at all. More striking even is the negative sign of n when the higher viscosities are taken into account. The mere attempt to define a *negative solvatation factor* is sufficient to demonstrate the physical impossibility of these results.

For solutions whose viscosities, for the same amount of solute, are still higher than in the preceding example, the factor n becomes negative for the whole range, while at the same time the general agreement is much improved.

As example we may take nitrocellulose in acetone (the first three sets of values in F. Baker's Table II):

	g. per 100 cc.	p	η	$\log \eta$
(1)	0·2212	0·2802	3·87	0·58912
(2)	0·5261	0·6647	18·73	1·27254
(3)	0·7922	0·9977	55·87	1·74720

If we again calculate the three constants from all three pairs of equations we find:

From (1) and (2)	$\theta = 2{\cdot}266$	$n = -28{\cdot}7$,
From (1) and (3)	$\theta = 2{\cdot}282$	$n = -31{\cdot}4$,
From (2) and (3)	$\theta = 2{\cdot}350$	$n = -35{\cdot}2$.

Here the constancy of θ and n, although far from perfect, is not unreasonably bad, especially if we consider that the assumption of a constant solvatation factor is not based on any known facts and is not inherently probable. The factor, however, is always negative and is thus devoid of any physical meaning. Nevertheless the values calculated from (2) and (3) may be used for calculating the next higher viscosity with reasonable accuracy.

If we take a further example showing still higher viscosity the results become much more erratic. The data are those for nitrocellulose in ether-alcohol (the first three values in Baker's Table XX).

	g. per 100 cc.	p	η	$\log \eta$
(1)	0·3284	0·4342	8·49	0·92880
(2)	0·5977	0·7882	26·33	1·42049
(3)	0·9230	1·214	114·50	2·05866

The constants obtained are:

From (1) and (2)	$\theta = 2{\cdot}75$	$n = -66{\cdot}6$,
From (1) and (3)	$\theta = 2{\cdot}50$	$n = -39{\cdot}1$,
From (2) and (3)	$\theta = 2{\cdot}08$	$n = -19{\cdot}7$.

Any discussion of these results is of course impossible, but the negative solvatation factors are striking.

A fourth example, in which the rise in viscosity is much more moderate, although still higher than with any protein, may be of interest. The data again refer to india rubber in benzene:

	g. in 100 cc.	p	η	$\log \eta$
(1)	0·25	0·281	1·6	0·20412
(2)	0·50	0·562	2·9	0·46240
(3)	1·00	1·124	16·6	1·22011

The two constants are:

From (1) and (2)	$\theta = 0{\cdot}644$	$n = 37{\cdot}6$,
From (1) and (3)	$\theta = 0{\cdot}654$	$n = 34{\cdot}4$,
From (2) and (3)	$\theta = 0{\cdot}666$	$n = 33{\cdot}6$.

In this case the constancy of both factors is fairly satisfactory and the value of n is positive throughout. Even if it were possible, which of course it is not, to disregard the negative factors found in the previous examples, the question would still have to be answered why a solvatation factor of 34 should be possible with one colloid, whereas a factor of 8·6 should be entirely incredible in another case.

Since the formula given by Arrhenius is apparently purely empirical and is not, as far as the author is aware, deduced from any physical considerations, a discussion is possible (and called for) only on purely mathematical grounds. The logarithm of the viscosity is in the formula equated with a product, one factor of which is a constant, θ, while the other, considered simply analytically, is a function of p and of a variable parameter, n. As the $\log \eta$ curves are very simple curves of a type strongly resembling in general habitus the curves of the family

$$y = \frac{100p}{100 - (n+1)\,p}$$

where n, as already explained, is a variable parameter, there is at least a strong probability that by a suitable choice of n a curve can be found which will coincide with a given $\log \eta$ curve over a considerable length, within the limits of experimental error, provided both are drawn to suitable scales. The latter condition of course finds its analytical expression in the proportionality factor θ. Although the factor n is introduced with a physical meaning, we have seen that negative factors, which have no possible physical meaning, result from the application of the formula to many cases, and such negative factors occasionally achieve the desired result, i.e. more or less close interpolation or approximation to experimental results.

The formula therefore seems to be a purely analytical device for representing a viscosity, or rather $\log \eta$, curve, the approximation being achieved by a factor n which in some cases is devoid of any physical interpretation. It is therefore certainly an unwarranted assumption that values of n, which are physically possible and happen to agree with a priori assumptions such as the very dubious parallel between hydrated salts and colloids which "swell strongly in water," necessarily have the physical

meaning ascribed to them, and that the higher values calculated with the aid of the author's formula are therefore necessarily incorrect.

It is only fair to add, in conclusion, that a difficulty also arises when an attempt is made to apply the author's viscosity formula to the examples treated above, or to highly emulsoid sols generally, a difficulty however which does not amount to a physical impossibility or even improbability. If the association factor is calculated say for the 0·5 % concentration of the first example ($\eta = 6{\cdot}8$) we find that about 62 % of the total volume of solvent is already associated with the solute. A 1 % sol would therefore be impossible, if the association factor were constant. There is certainly nothing inherently improbable about decreasing association with increasing concentration, and it is not difficult—as the author has found—to construct empirical functions connecting concentration and solvatation. As this is again a purely mathematical exercise affording no insight into the mechanism of association, the matter is more profitably left open until a rational solution of the whole problem, which is by no means hopeless, can be accomplished.

Summary.

By applying his logarithmic formula to the viscosities of protein sols as determined by Miss Chick, Arrhenius obtains hydratation factors ranging from 0·7 to 2·2. Since these figures are of the same order of magnitude as those found for hydrates of salts in solution, Arrhenius considers that they represent the actual conditions and that for the same reason the much higher factors calculated by applying Hatschek's formula are impossible. In the present paper Arrhenius's formula is applied to sols in organic solvents and it is shown that in several instances negative association factors result, which of course have no physical meaning. The conclusion is therefore drawn that Arrhenius's formula is a purely analytical interpolation formula and that the association factors calculated from it need not, even when they happen to be positive and therefore physically possible, have the physical meaning which he ascribes to them.

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XXIX. SOME EXPERIMENTS ON FACTORS CONCERNED IN THE FORMATION OF THROMBIN.

By HENRY HALLETT DALE and GEORGE STANLEY WALPOLE.

(*From the Department of Biochemistry and Pharmacology, Medical Research Committee.*)

(*Received July 21, 1916.*)

The experiments described in this paper were undertaken as part of an investigation into the causes of the toxicity acquired by serum from normal animals, when treated in certain ways. These toxic properties have been supposed by a number of investigators to be related to the phenomena of the anaphylactic reaction, and the name "anaphylatoxin" or "serotoxin" has been applied to the hypothetical toxic constituent. Some have supposed that the toxic substance is a protein cleavage-product; others that the toxicity is due to some undefined alteration in the physical properties of the colloidal system. We may remark that it is not as yet clear, that the toxic action produced by the various treatments, to which the serum has been exposed by different workers, is in all cases the same. Our attention was first directed to the toxic action, which fresh guinea-pig's serum very regularly acquires when shaken with about twice its volume of chloroform, as described in a series of papers by Jobling [1914] and his co-workers. We were struck by the conspicuous part played, in the toxic action of such a serum, by massive intravascular clotting. The occurrence of clots in the heart is mentioned, indeed, incidentally by these authors, but is evidently regarded by them as a subsidiary feature of the action. It may be so. We decided, however, to begin our proposed analysis of the action by examining this aspect of the effect, and were thus led to make a series of experiments on the phenomena of blood-clotting in vitro, and the effect, on the factors concerned, of treating plasma with chloroform, along similar lines to those which have been found to produce toxicity of sera.

A few preliminary experiments were made with mammalian serum and plasma. The investigation was complicated, however, by the necessity of

using a calcium precipitant to secure a plasma which did not coagulate spontaneously. We turned, therefore, to the plasma of the fowl, which several investigators have used on account of its stability when carefully prepared. The examination of the changes produced in it by treatment with chloroform necessitated the preparation of a series of the individual clotting-factors in as nearly pure a condition, in a physiological sense, as was practicable; and we were thereby at once involved in a consideration of different theories of blood-coagulation. For the mere definition of the factors concerned in normal clotting, and of the criteria of physiological purity to be applied to them, involves at least a provisional acceptance of one of the theories of their nature and function.

Our purpose in this preliminary paper, therefore, is to describe the methods which we have employed in preparing test-solutions of the clotting factors, and the information which we have obtained, by their use, concerning the processes occurring in a plasma treated with chloroform. The results so obtained seem to us incidentally to give very clear evidence against one recently propounded conception of the clotting-process. Later, when external conditions are more favourable to the steady pursuit of such an investigation, we hope to apply these methods and results to the study of induced toxicity of sera, and of the problem of anaphylaxis.

Theories of Blood Coagulation.

We are not immediately concerned with the mode of origin, or the precise nature of the interaction between the clotting-factors. So many reviews of the large literature of the subject have appeared, that we need only indicate briefly the main points of the two conceptions between which we attempt a decision. The principal current conception was present, in its essential features, in the theory finally put forward by Alexander Schmidt [1892, 1895], at the conclusion of his protracted and classical investigation; though in Schmidt's statement it was obscured by other conceptions which later work has failed to uphold. The theory, in its present relatively clear form, was put forward independently by Morawitz [1904] and by Fuld [1903], and numerous other investigators have contributed evidence as to the mode of origin and interaction of the factors concerned. The theory involves the interaction of three original factors, in addition to calcium ions, and the variety of the nomenclature used by different authors is somewhat bewildering. The factors are (1) fibrinogen, the soluble globulin of plasma which is converted into insoluble fibrin by the action of "thrombin" (fibrin ferment);

(2) an inactive precursor of thrombin, present in circulating blood and in stable plasma, which has been variously called plasmozyme, serozyme, thrombogen, and for which (following Mellanby [1908] and other recent writers) we use Schmidt's term "prothrombin," though this involves some restriction of Schmidt's application of the term; and (3) a substance obtainable from cellular tissues, or from blood-platelets, which in the presence of calcium ions brings about the conversion of the inactive "prothrombin" into the active thrombin. This substance was recognised by Schmidt, and by him called "zymoplastic substance." It corresponds to the "*A*-Fibrinogen" of Wooldridge, and has been variously termed thrombokinase (Morawitz), cytozyme [Fuld and Spiro, 1904], and, more recently, "thromboplastin" [Howell, 1910]. We use here, again following Mellanby, Morawitz's term "thrombokinase," or more briefly "kinase," for this factor.

An alternative conception, put forward in recent years by Howell [1911], and elaborated in a series of communications from his laboratory, differs from the foregoing in the importance attributed to "antithrombin." The presence in plasma or serum of factors inhibiting the coagulant effect of thrombin on fibrinogen had been recognised by a number of earlier investigators, and their action collectively attributed to "antithrombin." Howell supposes that prothrombin is converted into thrombin simply by the action of calcium, and that this action is prevented in circulating blood, or in stable avian or reptilian plasma, by the fact that prothrombin is associated with antithrombin. The rôle of the kinase (thromboplastin), according to Howell's view, is to dissociate this complex, by combining with the antithrombin, freeing the prothrombin to the action of the calcium ions.

It is clear that an acceptance of one or other of these theories is implicit in any definition of the clotting-factors, when an attempt is made to prepare them separately. Howell, for example, regards a preparation as pure prothrombin, in respect of its activity, if it fails to clot fibrinogen when added in the absence of calcium ions, but clots it promptly if an ionised calcium salt be added as well. He would regard a prothrombin, which fails to produce clotting under such conditions, and needs the further addition of kinase for its activation, as contaminated with antithrombin. Adherents of what we may, for brevity's sake, term the Morawitz theory would conclude, on the other hand, that the activation of Howell's "prothrombin" by calcium ions alone was clear proof of its contamination with kinase; it might even be kinase only, without any prothrombin, if the fibrinogen used had not been carefully freed from the prothrombin which is readily precipitated with it.

Preparation and Testing of Reagents.

1. *Fibrinogen.* The only difficulty in preparing an adequate fibrinogen solution is to get it absolutely free from prothrombin. Prothrombin is either itself a euglobulin, or is closely associated with one. Mellanby, indeed, appears to regard fibrinogen and prothrombin as so closely associated, that he frankly abandons the possibility of separating them, and uses as "fibrinogen" a preparation from which prothrombin can be separated by clotting the fibrinogen with thrombin.

This practice has the disadvantage that the decision, whether a given solution contains thrombin or kinase, is frequently a matter of indirect inference; and when both are present in the preparation being examined, the presence of adventitious prothrombin in the reagent fibrinogen makes the interpretation of results excessively complicated and difficult.

To obtain a fibrinogen free from this defect we used a device indicated by Bordet and Delâge [1912]. These authors showed that an oxalated plasma, exposed to the adsorbing influence of precipitated barium sulphate, becomes indefinitely stable on recalcification, and will no longer clot when kinase is added in any amount, but yields a good clot with thrombin.

A plasma so treated ought, therefore, to yield a fibrinogen free from prothrombin. We find that it does so. A fair amount of the fibrinogen is apparently lost on the adsorbent barium sulphate; but enough remains to make easy the preparation of a strong fibrinogen solution. As a precipitant of fibrinogen we have used an equal volume of saturated sodium chloride, following Hammarsten [1879]. Mellanby suggests that Schmidt's earlier method of precipitating by dilution and acidification gives a less denaturated product. Our experience with oxalated rabbit-plasmata is quite contrary to this. We found that dilution and acidulation, even with the greatest care to attain the optimum reaction, produced in such plasmata a scanty precipitate, in an inconveniently large volume of fluid. This precipitate, collected by the centrifuge, was seldom perfectly soluble in neutral saline, and became less so if it were taken up in dilute sodium carbonate, and reprecipitated by dilution and acidulation. We therefore adopted the following routine procedure, which is similar to that employed by Howell, except that the plasma was freed completely from prothrombin before precipitation.

Rabbits were etherised and bled from the carotid artery, through a paraffined cannula, into large centrifuge tubes. 10 cc. of a watery solution containing 1 % potassium oxalate with 0·9 % sodium chloride were put into

each tube as a preliminary. If the volume of blood obtained exceeded 90 cc., an extra amount of the oxalate solution was added as soon as the 100 cc. mark was attained. The oxalated blood was cooled and thoroughly centrifuged. The plasma was carefully pipetted off, rejecting the layer immediately in contact with the corpuscles and platelets.

A standard suspension of barium sulphate was prepared as follows. 31·5 g. of barium chloride were precipitated with a calculated slight excess of sodium sulphate, and washed repeatedly with 1 % sodium chloride solution by decantation, until the washings were free from sulphate. The total volume was then made up with 1 % sodium chloride to 200 cc. To every four volumes of oxalated plasma were added three volumes of the well-shaken suspension of barium sulphate.

The mixture of plasma and barium sulphate was well stirred, and allowed to stand for some hours—usually over night. It was then centrifuged clear, and the plasma poured off from the heavy barium sulphate. To each volume of the now diluted plasma was added an equal volume of a saturated solution of pure sodium chloride. Precipitation occurs rather slowly. As soon as separation was complete—i.e. usually after about an hour—the precipitate was separated with the help of the centrifuge, dissolved in 2 % sodium chloride, and again precipitated with an equal volume of saturated sodium chloride. The precipitate was collected again and dissolved in a convenient volume of 2 % sodium chloride. A trace of sodium hydrate was added if necessary to assist solution, and a measured equivalent quantity of standard hydrochloric acid then added to neutralise. The solution was centrifuged clear from any flakes of denaturated material, if such were present, and finally diluted with water to a content of 1 % sodium chloride. Such a solution is, apparently, indefinitely stable if kept cold. It yields a hard clot in a few minutes if a drop of active thrombin be added to a few drops of the fluid. The following represents the usual test and its result:

Fibrinogen solution 5 drops + 1 % sodium chloride 10 drops + thrombin solution 1 drop. Put into bath at 37°.—Clotted firmly in $2\frac{1}{2}$ minutes.

[In recording subsequent experiments we adopt the convention of using only the name of a clotting factor to indicate the stock solution of it here described. The figure following it indicates the number of drops added. Saline = 1 % pure sodium chloride. Calcium = 1 % calcium chloride. All determinations were done in a water-bath at 37°.]

On the other hand, neither of the other clotting-factors can be made to clot such fibrinogen solutions when added separately. With either prothrombin

and calcium, or kinase and calcium, using these terms as defined below, no trace of clotting could be observed, however long the incubation was continued.

2. *Prothrombin.* It is evident that the definition of "prothrombin" involves adhesion to one or the other of the two theories mentioned above. Since one of our objects was to discover additional facts which would not, as are so many of those hitherto available, be explicable on either theory, we were obliged to make our definition in a sense experimental. Previous workers have obtained prothrombin preparations, which either failed altogether to clot fibrinogen with calcium only, or did so extremely slowly, and had their action enormously accelerated by kinase. On the Morawitz theory, such preparations are prothrombin, either pure, or contaminated by traces of thrombin. On Howell's view, they are prothrombin which cannot become thrombin in the presence of calcium, on account of associated antithrombin. If, therefore, we could prepare a prothrombin which showed no sign of activation with calcium alone, which rapidly yielded thrombin when kinase in addition was added, and which contained no demonstrable antithrombin, we should have good reason for not accepting Howell's view. There would remain, in regard to that view, only the duty of showing how Howell's evidence in its favour could be explained along other lines. We are convinced that we have on numerous occasions obtained such preparations of prothrombin, practically free alike from thrombin and from antithrombin, not activated by calcium alone, but readily by kinase in addition. One method of obtaining such is the treatment of fowl's plasma with chloroform under appropriate conditions, which we discuss in a later section. Such preparations, however, are very unstable, for reasons which we there discuss. Moreover the conditions governing their production are so complex, that there is an element of uncertainty in the method, as we shall also show later.

Mellanby's method of preparing prothrombin from fowl's plasma is admirable as a practical process. From the controversial point of view it has two defects. It is difficult to add just so much thrombin that all the fibrinogen is removed from the solution containing it and prothrombin, and no excess of thrombin left. One easily obtains prothrombin with some fibrinogen, still capable of yielding a weak clot when kinase and calcium are added; and one easily obtains prothrombin with so little thrombin, that a quantity of the preparation, which clots fibrinogen in a few seconds after treatment with kinase and calcium, will clot it only after half an hour or

more, when added with calcium alone. But this presence of a trace of thrombin gives to the action of the kinase the appearance of a mere acceleration. And this is important, as being capable of reconciliation with Howell's ideas; since Mellanby's method unmodified gives no guarantee against the presence of some antithrombin, albeit in traces which would not seem to us significant. By a slight modification, however, prothrombin, free alike from perceptible traces of thrombin or antithrombin, can be obtained.

In a fowl anaesthetised with ether the main artery of the leg is exposed, isolated, tied, and clamped proximally to the ligature with bull-dog forceps. The surface is washed carefully with oxalated saline, a cut with clean scissors made between ligature and bull-dog clamp, and the interior and cut surface washed through a capillary pipette with the oxalated saline. A clean glass cannula is then tied in and the blood allowed to flow straight into a large centrifuge tube. In this the blood is rapidly cooled to nearly 0° C., and is then centrifuged for about 15 minutes at 4000 revolutions per minute with a mean radius of 20 cm. The clear plasma is pipetted off. If stored cold, such a plasma seems to remain indefinitely stable. It does not clot at 37° C. within any reasonably limited time of observation. If diluted with two or three volumes of water, it usually clots after some hours.

A mixture of fibrinogen and prothrombin can be precipitated from such a plasma, as Mellanby describes, by diluting with twenty volumes of water and bringing the reaction to the isoelectric point of the globulins by adding acid. The association of fibrinogen and prothrombin in this precipitate is regarded by Mellanby as indicating their association as a preformed complex in the plasma. The force of this suggestion seems to us to be weakened by certain observations of our own. We have already described how fibrinogen can be prepared free from prothrombin by adsorbing the latter with barium sulphate. Mellanby showed that prothrombin was left in solution when a fibrinogen-prothrombin mixture was clotted with thrombin. We have now to add evidence that the two can be separated by fractional precipitation, brought about by careful reduction of electrolyte content. It seems to us, therefore, that the regularity with which fibrinogen and prothrombin occur together, in precipitates obtained by diluting and neutralising plasma, merely signifies that prothrombin has the solubilities of a euglobulin, like fibrinogen. It appears, however, to be soluble in a weaker solution of pure sodium chloride than is required to dissolve fibrinogen. The details of an actual preparation will explain the point.

50 cc. of clear, fresh fowl's plasma were diluted with 1 litre of distilled

water. One-tenth normal acetic acid was then added till maximum precipitation was obtained, 15·75 cc. being required. The precipitate was collected by the centrifuge, rubbed up with distilled water and again separated. It was then dissolved in 18 cc. of 1 % pure sodium chloride solution. The solution still showed a trace of the yellow colour of the original plasma. 11 cc. of it were poured into a glass vessel and warmed to 37° in a water-bath. A stock solution of thrombin was added, one drop every half-hour, till a weak clot began to form. The solution was then removed and allowed to stand at laboratory temperature over night. Next morning the now solid clot was broken up, and the serum removed. The object was to remove most of the fibrinogen without leaving any excess of thrombin in the serum. This was apparently attained, since five drops of the serum failed to clot five drops of fibrinogen solution in several hours. The serum, however, still contained some fibrinogen, since a sample yielded a soft clot in a few minutes on adding kinase and calcium. It also contained a remnant of "antithrombin," as we shall show, and was, therefore, further treated as follows. It was first dialysed for 4 hours against distilled water in a collodion tube. A fairly copious precipitate formed, and a specimen of the supernatant fluid on testing was found to be relatively weak in prothrombin, and to possess a weak, but distinct antithrombic action. The contents of the dialysing tube were therefore poured out and spun in the centrifuge. The supernatant fluid was then poured off, being retained for a test of its antithrombic action, and the precipitate was dissolved in 10 cc. of 1 % sodium chloride. On dilution with 10 cc. of distilled water no precipitation occurred; but on adding a further 10 cc., reducing the sodium chloride content to 0·33 %, a flocculent precipitate was formed. This contained practically all the fibrinogen; for the supernatant fluid, separated clear by the centrifuge, no longer gave any trace of clot with kinase and calcium, though it contained a powerful prothrombin. This fluid was, indeed, such a powerful prothrombin that it was diluted with twice its volume of 1 % sodium chloride, for routine use. Experimental details may be given of the tests made of its properties, since the results seem to us to constitute a very clear refutation of Howell's theory. On this theory, the solution, having all the properties of prothrombin in a high degree, should (*a*) yield thrombin on treating with calcium ions alone, or (*b*) contain at least a definitely recognisable amount of antithrombin. We find no trace of either action.

EXPERIMENT 89 (*b*).

Test of solution (*S*) *for thrombin.*

(*a*) Preformed.

Fibrinogen 5 + saline 7 + *S* 5.—No clot in 1 hour.

(*b*) Formed in presence of calcium ions.

Fibrinogen 5 + saline 7 + calcium 1 + *S* 5.—No clot in 1 hour.

Test for prothrombin.

S 5 + calcium 1 + kinase 2—incubated together 10 minutes; then added: Fibrinogen 5 + saline 7.—Hard clot in 30 seconds.

Test for "antithrombin." In using, for convenience, the term "antithrombin," we have no intention of committing ourselves to the assumption that plasma or serum contains a definite substance, having a specific affinity for thrombin, and a neutralising effect on its action. What is definitely known is, that plasma and serum contain some substance or substances which hinder and delay clotting. Some may, and probably do, act by becoming physically associated with the thrombin; others may hinder the gelation of the fibrin. Howell seems to assume that the substance, which delays the action of formed thrombin, is the same as that which, on his theory, hinders the combination of prothrombin with calcium to form thrombin. The reason for this assumption is not clear to us, but a good deal of Howell's evidence is directed to showing that kinase weakens the effect of the factor delaying the action of formed thrombin. We are, therefore, entitled to suppose that the presence of this factor in a prothrombin, which is not activated by calcium ions alone, is essential to his argument. This factor has been shown by Howell, among others, to be stable at 60°; and since our thrombin contains traces of kinase, all solutions containing prothrombin had to be freed from the latter by heating to this temperature before their antithrombic value could be fairly tested. In comparative tests, like the following, the preparations were heated side by side, in the same water-bath, being immersed and removed together. The thrombin solution was diluted so that one drop, added to the standard fibrinogen dilution, caused clotting in about 10 minutes. In addition to the reagents already specified, we prepared heated fowl plasma, and heated solution of prothrombin (*S*), the heating being carried out by immersion in a water-bath at 60° for 20 minutes. The following are the tests and their results:

EXPERIMENT 89 (*b*) (*continued*).

1. (Control.) Fibrinogen 5 + saline 12 + diluted thrombin 1.—Clotted in 11 minutes.

2. Fibrinogen 5 + heated prothrombin 7 + saline 5 + thrombin 1.—Clotted in 11 minutes.

3. Fibrinogen 5 + heated plasma 7 + saline 5 + diluted thrombin 1.—No clot in 3 hours. Found clotted next morning—16 hours.

It is quite clear, then, that the prothrombin solutions contain no recognisable fraction of the substance inhibiting thrombin action, which can be demonstrated in the original plasma. When the original fibrinogen + prothrombin precipitate, obtained according to Mellanby's directions, was purified by redissolving, and then again precipitating by dialysis, the supernatant fluid from the dialyser was found to contain a trace of the antithrombin, as the following results show. The solutions were heated together to 60° as before, but a thrombin of twice the strength was used in this experiment.

1. (Control.) Fibrinogen 5 + saline 12 + thrombin 1.—Clotted hard in 5½ minutes.

2. Fibrinogen 5 + saline 5 + heated prothrombin 7 + thrombin 1.—Clotted hard in 5½ minutes.

3. Fibrinogen 5 + saline 5 + heated supernatant fluid from dialyser 7 + thrombin 1.—This also showed signs of clotting after 5½ minutes in the thermostat, but the clot was soft, and was not properly hard even in 1 hour.

4. Fibrinogen 5 + saline 5 + heated plasma 7 + thrombin 1.—An extremely soft clot began to form after 11 minutes, and was still soft after 1 hour.

We have found, in experiments on the effect of varying quantities of antithrombin, that a delay of the firm setting of the clot appears with amounts which no longer clearly delay its first appearance. We feel entitled, therefore, to conclude that small quantities of the antithrombin of plasma adhere to the precipitate of fibrinogen and prothrombin, obtained from the plasma by dilution and adjustment of reaction. These, however, can be removed by reprecipitating the mixed euglobulins by dialysis. There is no indication of the formation of thrombin from the prothrombin during the process, nor on the subsequent addition of calcium salts. On the subsequent addition of kinase, thrombin is formed, and, if fibrinogen is present, this is clotted. A further series of tests may be quoted, to illustrate this last point, together with the other properties of a preparation of prothrombin made according

to Mellanby's directions, without further purification. This specimen, when freshly prepared, contained some thrombin, and probably, from previous experience, traces of antithrombin. After keeping in the cold for a few days, no thrombin or antithrombin could be detected in it. Physiologically it was now a pure and powerful prothrombin, though its activity in this respect had also undergone slight decay.

EXPERIMENT 88.

1. *Test for prothrombin.*

Solution 5 + calcium 1 + kinase 2.—Incubated 10 minutes.

Then added fibrinogen 5 + saline 7.—Clotted in 1½ minutes.

2. *Test for preformed thrombin.*

Fibrinogen 5 + saline 10 + solution 5.—No clot in 3 hours.

3. *Test for formation of thrombin with calcium ions, but no kinase.*

Fibrinogen 5 + saline 7 + solution 5 + calcium 1.—No clot in 1 hour.

Then added: kinase 2—clotting began in 12 minutes, and was complete in 17 minutes.

4. *Test for antithrombin,* in solution heated to 60° for 8 minutes.

As a control we used, in this case, a fowl-plasma which had lost some of its antithrombin value by keeping. It was heated to 60° for 20 minutes.

Control 1. Fibrinogen 5 + saline 12 + diluted thrombin 1.—Clotted hard in 18 minutes.

Control 2. Fibrinogen 5 + heated fowl-plasma 7 + saline 5 + diluted thrombin 1.—Weak, soft clot in 1½ hours.

Test. Fibrinogen 5 + heated solution 7 + saline 5 + diluted thrombin 1. —Clotted hard in 18 minutes.

Here, then, we have again a prothrombin, which contains no demonstrable antithrombin. It is activated by kinase in the presence of calcium to a powerful thrombin, but even prolonged incubation with calcium alone fails to produce any thrombin from it; the only effect of such long incubation, with calcium and fibrinogen, is an apparent delay in the subsequent activating effect of kinase.

3. *Thrombin.* We have not prepared a pure thrombin solution. We required thrombin mainly as a test for the continued presence of fibrinogen in a solution, which has failed to clot with other reagents, and for testing the antithrombic effect of various preparations. Provided the latter contained

no prothrombin, which was excluded by previous heating, the existence in our thrombin of a trace of kinase, which it undoubtedly contained, was immaterial. It was prepared by whipping the fibrin from rabbit's blood, as shed from the artery; washing the fibrin till white by kneading with water; and then extracting with 8 % sodium chloride, according to Howell's modification of Gamgee's method. The thrombin so obtained was, as a rule, unnecessarily powerful for general use. It was usually kept in the strong solution, and portions diluted as required. Diluted with an equal volume of water, it gave a solution of which one drop caused firm clotting of our standard mixture of 5 drops of fibrinogen + 10 drops of saline in 2 or 3 minutes. For detecting small amounts of antithrombin it was diluted further, so that one drop clotted such a mixture in 10 to 20 minutes. After heating to 60° the strong solution showed no thrombic action, failing to clot pure fibrinogen solutions in any period. A weak activating (kinasic) effect on prothrombin in the presence of calcium remained after such treatment.

4. *Thrombokinase.* As a source of kinase, following Mellanby's recommendation, we used the fowl's testis, stripping off the capsule with its blood-vessels. The pulp was allowed to stand at room temperature for a day, and then treated by Howell's method—i.e. it was ground up with sand, dried, and then rubbed up with glycerol. The mixture was centrifuged and the turbid supernatant fluid, unfiltered, was diluted as required with two volumes of distilled water. Such dilutions appeared to keep well for a week or two, and then suddenly to lose their effect. The strong glycerol extract seemed to be indefinitely stable.

The Effect of Chloroform on Plasma, etc.

As stated in the introduction, our ultimate object is a closer analysis of the effect of extraction with chloroform, and other forms of treatment, in imparting toxicity to sera. A more immediate interest was given to the matter, from our present point of view, by the discovery that shaking with chloroform, which has been shown by Jobling and his associates to destroy antitrypsin in serum, very rapidly and completely destroys the antithrombic action of serum or plasma. Since we first observed this effect, a paper has appeared by Minot [1915], in which he shows that shaking oxalated mammalian plasma with a little chloroform will frequently induce clotting. He, rightly in our opinion, attributes this to the effect of some preformed thrombin, which is able to act when the antithrombin has been destroyed.

He further reaches the conclusion that chloroform does not convert prothrombin into thrombin as Howell [1910] had originally supposed. Minot's meaning seems to be that the mere removal of antithrombin, in the absence of calcium, does not activate Howell's preparation of prothrombin, of which we consider the properties in another section.

Our own first experiments with fowl's plasma gave results which, at first sight, seemed to be strongly in favour of Howell's theory. On shaking a small volume of fowl's plasma with twice its volume of chloroform, keeping the mixture warm in a water-bath at 37°, we found that, after about 10 minutes, the emulsion suddenly acquired a semi-solid consistency, as though clotting had occurred. The now permanent emulsion was kept at 37° for about an hour, and then removed to centrifuge tubes, and spun rapidly for about 15 minutes. The clear, supernatant watery layer was pipetted off and tested. It was found to possess a very powerful thrombin action. After dilution to five times its volume, one drop clotted the standard fibrinogen mixture in 2 minutes. After heating to 60° the preparation was found to be devoid of antithrombic action. This observation lends itself readily to interpretation in accordance with Howell's views. Fowl's plasma is known to contain much prothrombin, and a good deal of antithrombin. According to Howell, it is this latter which prevents the prothrombin from becoming thrombin by union with calcium ions. We have removed the antithrombin by chloroform treatment, and thrombin is formed. To that extent, therefore, the expectation is fulfilled. A few further experiments, however, sufficed to dispel this plausible interpretation of the effect.

In the first place, the fluid obtained by chloroform treatment of the fowl-plasma, readily produced clotting of many times its own volume of the plasma from which it was produced, or of any other stable fowl-plasma. For example, the following mixtures were placed in the bath at 37°, with results as indicated:

EXPERIMENTS 48 AND 49.

1. Fowl's plasma—5 drops.

Saline 1 drop.—No clot in 24 hours.

2. Fowl's plasma—5 drops.

Chloroform-treated fowl's plasma (as above)—1 drop.—Clotted firmly in $1\frac{1}{2}$ minutes.

3. Fowl's plasma—10 drops.

Chloroform-treated fowl's plasma—1 drop.—Clotted firmly in $2\frac{1}{2}$ minutes.

If the activation of the plasma were to be explained as the result of removal

of antithrombin, the result in tube No. 3 would oblige us to conclude, that the loss of not more than 1/11 of the total antithrombin present in a sample of the plasma allows the formation of sufficient thrombin to clot the whole in a few minutes. And this conclusion would be wholly at variance with all other experience of the neutralising effect of antithrombin on the action of thrombin. If we are dealing with a sample of antithrombin, of which eleven drops so delay the action of a given dose of thrombin that the clotting-time is lengthened from, say, 10 minutes to 5 hours, then we shall find that ten drops of the same antithrombin prolong the clotting-time to $4\frac{1}{2}$ or $4\frac{3}{4}$ hours, perhaps; but that clotting should occur in $2\frac{1}{2}$ minutes with ten drops, and be altogether inhibited by eleven drops of an antithrombic preparation from plasma or serum, is an impossible result.

It might possibly be urged that this argument only applies to the free antithrombic constituent of serum or plasma, which survives heating for some minutes to 60°; and that the possibility remains of prothrombin being associated in plasma with some other thermolabile inhibitory factor, which is similarly liable to the destructive action of chloroform. Such a suggestion, however, has no direct bearing on either the evidence or the theory which we are discussing. The only factor, of whose destruction by chloroform we have direct evidence, is thermostable; and it is an essential feature of Howell's view, if we rightly understand it, that it postulates an association between prothrombin and this thermostable antithrombin, which is not readily destroyed at 60°; and that it attributes to the effect of this factor the inability of calcium alone to activate a prothrombin preparation. It is easy, moreover, to dispose of the suggestion by experiment. If the activation of fowl-plasma by chloroform were due to the removal of an antithrombic factor of any kind, it should clearly be possible to activate, by similar treatment, a prothrombin solution prepared from that plasma. This we have repeatedly attempted, with no hint of success. If a prothrombin, prepared from fowl's plasma according to the indications of a previous section, be shaken with chloroform, either in the cold or at 37°, the prothrombin is fairly rapidly destroyed, but no trace of thrombin appears. As long as activity of any kind remains, it is that of prothrombin—i.e. of a substance which clots fibrinogen only after treatment with calcium and kinase, and never with calcium alone.

We may conclude, then, that the activation of fowl-plasma by chloroform is due neither to the removal of antithrombin, revealing a previously neutralised thrombin, nor to direct action of the chloroform on the prothrombin. To elucidate the nature of the action we decided to examine the different

layers of the plasma-chloroform emulsion, after different periods of treatment at varying temperatures, for the presence of the several known clotting-factors, using the set of reagents previously described.

It should be stated at once that the results obtained, though all pointing to the same conclusion, showed considerable variation. After shaking a specimen of stable fowl-plasma for a given time and at a given temperature with chloroform, separating the emulsion by the centrifuge, and then examining separately the clear supernatant watery layer, and the emulsion of more or less denaturated material separating with the chloroform-layer, we found it impossible to prophesy, in any given case, the nature and distribution of the clotting factors which we should find. It varied, not only with the plasma from different fowls, but with the same sample of plasma, after different periods of storage in the cold-room. The reason of this variability in the result will be sufficiently clear, when we have described the experiments which demonstrate the complexity of the processes at work. We mention it here in explanation of the fact, that we choose, for extended description, experiments in which the conditions made it possible to follow clearly the stages of the production of thrombin. No results were obtained which were inconsistent with the conception thus obtained; but many in which intermediate stages were missed, or in which, owing to changes which will later become clear, the process was incomplete.

The procedure adopted was, in its general features, identical in all cases. To one volume of the plasma we added two volumes of chloroform. It is quite probable that a much smaller volume would have effected the changes which we describe; but to experiment with different proportions would have made the number of tests required unmanageably great. After the two had been shaken together under the conditions of the particular experiment, the mixture was thoroughly centrifuged. The clear watery layer could then be poured off clean from the stiff emulsion which formed the upper portion of the chloroform-layer. The watery layer was, if necessary, freed from traces of dissolved chloroform by blowing air over it. The chloroform-layer was then stirred, and the free, fluid chloroform poured away. The curdy emulsion left was then rubbed up with a volume of 1 % sodium chloride solution equal to twice the volume of plasma taken and over this a vigorous stream of air was blown until all chloroform was removed. Part of the material passed into solution during this process; part, in most cases, was rendered permanently insoluble by the denaturating effect of the chloroform.

We had, then, for examination the supernatant watery layer, and the

saline extract of the chloroform-layer. These will be referred to as "watery layer" and "chloroform-layer" respectively.

A preliminary, incomplete experiment may first be quoted.

EXPERIMENT 52.

A sample of fowl-plasma was shaken with chloroform till a permanent emulsion was formed, and this was left over night at laboratory temperature. Next morning the mixture was centrifuged and the two layers examined. The watery layer had a rather weak thrombin-action, as the following experiment shows:

Fibrinogen 5 + saline 5 + watery layer 1.—Clotted in 12 minutes.

The chloroform-layer, on the other hand, was much weaker in this respect:

Fibrinogen 5 + saline 5 + chloroform-layer 1.—Clotted in 50 minutes.

But the chloroform-layer, though having but weak thrombin action, revealed itself, on incubation, with prothrombin and calcium, as an effective kinase.

Prothrombin 5 + calcium 1 + chloroform-layer 2, incubated for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 4 minutes.

Here, then, was an indication that the chloroform treatment produces from the stable plasma something which functions as a kinase. In this experiment no attempt was made to study its distribution between the layers; it was merely shown that it was present in the chloroform-layer, which contained little thrombin. We shall show, by quoting other experiments, that this distribution between the layers is very variable. Several of the earliest of our complete experiments gave the impression that the kinase, after the centrifuging of the emulsion, was practically limited to the chloroform-layer. Later, however, we met with cases in which, after apparently identical treatment of a plasma, the watery layer had a very high kinasic value, while that of the chloroform-layer was hardly perceptible. We shall discuss the probable meaning of these discrepancies after illustrating them. For the present, the point of importance is that the chloroform-treatment caused the appearance of kinase in plasma, which certainly did not contain it before, since the untreated plasma was indefinitely stable even at 37°. We may add at once, also, that after treatment with chloroform at 37° for several days, kinase is the sole clotting-factor which can be discovered in either the watery layer, or in what remnant of water-soluble constituents the chloroform-layer then contains. In one such case the incubation was

carried on from a Saturday afternoon to the following Monday morning. After centrifuging, the watery layer was tested as follows:

1. *Test for thrombin.*

Fibrinogen 5 + saline 10 + watery layer 4.—No trace of clotting in 1 hour.

One drop of a known thrombin was then added to the tube and produced a firm clot in a few minutes. We may conclude, then, that the fluid under examination contained no thrombin.

2. *Tests for kinase.*

Prothrombin 5 + calcium 1 + watery layer 2, incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted firmly in less than 30 seconds.

The clotting-time being so short, the test was repeated, exactly as above, using, however, the watery layer diluted, first four times, and then twenty times. The clotting times were 2 minutes and 16 minutes respectively. Clearly, then, we were left with a very powerfully kinasic solution, while all thrombin, and necessarily all prothrombin, had vanished.

This discovery, of the formation of kinase in plasma treated with chloroform, at once suggested an explanation of the conversion of prothrombin into thrombin in a plasma so treated.

It will be clear that the combination of effects, thus demonstrated as resulting from the action of chloroform, gives us another opportunity of putting to the test the rival theories with which we are here concerned. On the Morawitz-theory the formation of thrombin is obviously explicable by the action of the freshly-liberated kinase on the prothrombin, in the presence of calcium. On Howell's theory the disappearance of the antithrombin would be the decisive feature of the action, the appearance of kinase only having significance, in so far as it assisted this. It occurred to us that a clear indication, in support of one or the other of these views, might be obtained by carrying out the chloroform treatment in the absence of calcium. It seemed probable that antithrombin would still be destroyed, and kinase still formed under such conditions, though activation of prothrombin would not take place till calcium was added. If, then, we could effect a separation of the prothrombin and the newly-formed kinase by the centrifuge, the effect of adding calcium would give us a definite answer; for on Howell's theory the layer containing antithrombin-free prothrombin should form thrombin with the calcium only, while on the Morawitz theory, admixture with the kinase-containing layer should be necessary before activation

occurred. All the conditions indicated have been realised in a series of experiments, and the result seems to us quite decisively against the theory of Howell, and in favour of that of Morawitz.

We may quote first an experiment on the action of chloroform on a further sample of the plasma used in Experiment 52.

EXPERIMENT 53.

To 15 cc. of plasma was added 1·5 cc. of 1 % potassium oxalate. 30 cc. of chloroform were then added, and the whole shaken mechanically at laboratory temperature for 1 hour. It was then thoroughly centrifuged and the two layers examined separately as described above.

Tests of the watery layer.

(1) *For thrombin.*

(*a*) Fibrinogen 5 + saline 10 + watery layer 5.—No clot after 2½ hours.

(*b*) Fibrinogen 5 + saline 10 + calcium 1 + watery layer 5.—No clot after 2½ hours.

Therefore no thrombin was present, and none was formed on addition of sufficient calcium chloride to remove excess of oxalate, and secure the presence of free calcium ions.

On adding two drops of kinase to each of the above tubes, at the end of 2½ hours, (*a*) showed no sign of clotting in a further hour, while (*b*) clotted in about 12 minutes—thus showing that it contained an effective amount of free calcium, as well as prothrombin.

(2) *For prothrombin.*

Watery layer 5 + calcium 1 + kinase 2—incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 4 minutes.

Therefore a moderate amount of prothrombin was present; and since, as shown under (1), no thrombin was formed with calcium alone, no intrinsic kinase can have been present in the watery layer.

(3) *For antithrombin.*

A weak thrombin was used, of which four drops clotted the standard fibrinogen dilution only after 10 minutes.

(*a*) Fibrinogen 5 + saline 11 + thrombin 4 + calcium 1.—Clotted in 10 minutes.

(*b*) Fibrinogen 5 + heated watery layer 8 + saline 3 + calcium 1 + thrombin 4.—Clotted hard in 14 minutes.

(*c*) Fibrinogen 5 + heated fowl-plasma 8 + saline 3 + calcium 1 + thrombin 4.—*Not* clotted in 100 minutes.

The trivial delay of clotting, from 10 to 14 minutes, by the heated watery layer cannot be regarded as indicating the presence of antithrombin. It is an effect such as might be produced by a slight difference in saline concentration, or slight increase of the alkalinity of the mixture. It will be noted that, under the same conditions, the heated original plasma lengthened the clotting-time to over 100 minutes—beyond which period the observation was not continued.

Tests of the chloroform layer.

1. *Tests for thrombin.*

(*a*) Fibrinogen 5 + saline 8 + chloroform-layer 4.—No clot in 30 minutes.

(*b*) Fibrinogen 5 + saline 8 + chloroform-layer 4 + calcium 1.—No clot in 30 minutes.

Therefore no thrombin was present, and none was formed in the presence of calcium.

2. *Test for kinase.*

Prothrombin 5 + calcium 1 + chloroform-layer 4, incubated together for 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted in 2 minutes.

Since, therefore, this layer contained plenty of kinase, it is clear that it contained no prothrombin, since the addition of calcium alone (under 1) did not produce any thrombin from it.

We have, therefore, in this experiment, by treating oxalated fowl-plasma with chloroform, produced kinase, which, however, in the absence of calcium, could not activate the prothrombin. Fortunately for the purpose of our enquiry, the kinase, in this instance, is found, after the emulsion has been centrifuged, to be limited to the chloroform-layer. The watery layer contains an adequate prothrombin, free from clearly recognisable antithrombin. Yet this prothrombin is not activated by treatment with calcium only; kinase in addition activates it promptly. We are entitled to conclude, therefore, that the formation of thrombin, when fowl-plasma, containing its normal calcium, is shaken with chloroform, is due to the production of kinase, which secondarily activates the prothrombin. The disappearance of antithrombin is an accidental accompaniment, not an essential feature of the process.

The clean separation of the prothrombin and kinase by the centrifuge, as might be expected, was not seen in every experiment of the kind. We may summarise a few of the varieties of result obtained in a long series of experiments, by indicating once for all that the tests for the different clotting-factors were made according to the following scheme:

Test for thrombin.

Fibrinogen 5 + saline 10 + specimen 4.

Test for prothrombin.

Specimen 5 + calcium 1 + kinase 2. After 10 minutes at 37°, add fibrinogen 5 + saline 5.

Test for kinase.

Prothrombin 5 + calcium 1 + specimen 2, and then as for prothrombin.

Test for antithrombin (on solution heated to 60°).

Fibrinogen 5 + specimen 7 + saline 5 + thrombin 1. (If the specimen was oxalated, calcium was added as well.)

EXPERIMENT 57.

A sample of the same fowl-plasma as used for Expts. 52 and 53 was oxalated, and then dialysed under pressure against distilled water, till all excess of oxalate was removed. It was brought back to the original volume with 1 % NaCl, and then centrifuged clear. It showed no tendency to spontaneous coagulation, even on adding calcium. It was treated with chloroform for half-an-hour, being kept ice-cold during the treatment. The watery layer contained no preformed thrombin, but, when calcium was added, it clotted fibrinogen after half-an-hour. If kinase were added as well (test for prothrombin) the combination clotted in 30 seconds. This watery layer, however, contained no trace of demonstrable antithrombin. The tube containing it clotted simultaneously, in 4 minutes, with the control tube containing an equal addition of saline; while the tube to which the oxalated and dialysed plasma, heated in parallel, was added, began to show a weak clot only after 53 minutes. The fact that the watery layer showed a trace of activation with calcium only could be fairly attributed to its containing a trace of kinase with its prothrombin. This view was supported by the discovery of kinase in the chloroform-layer. When four drops of the extract from the chloroform-layer were added to five of the watery layer, and the mixture incubated with calcium, it clotted fibrinogen in 9 minutes.

EXPERIMENT 62.

A sample of plasma from another fowl was oxalated, and was treated with chloroform in the cold for 15 minutes. A non-oxalated sample was treated in exactly the same way, and the two watery and chloroform-layers obtained were subject to a parallel series of tests. The watery layers of both contained abundant prothrombin, clotting fibrinogen in 15 seconds after preliminary treatment with kinase and calcium. The oxalated sample contained no thrombin, but a weak thrombin was present in that treated without oxalate (clot in 22 minutes). The chloroform-layers showed a stronger thrombin (6 minutes) in the case of the non-oxalated, none in that of the oxalated sample. Both contained kinase and prothrombin, so that the chloroform-layers, in this experiment presented a rather complex problem.

No antithrombin could be detected in either layer, with either sample.

EXPERIMENT 65.

The same plasma as above was treated, with and without oxalate, with chloroform for $1\frac{1}{4}$ hours at 37°. The watery layers were tested for thrombin, with the result that the sample without oxalate caused clotting in 1 minute, while the oxalated sample had produced no clot in an hour. Neither sample contained any antithrombin, clotting occurring simultaneously in the control, and in the tube containing the two samples. The time in each case was 3 minutes, and the three clots were indistinguishable in firmness. In a fourth tube, with the heated original plasma, a weak clot began to form after 30 minutes, and was still soft after several hours. The oxalated sample contained a fairly active prothrombin, causing clotting in $2\frac{1}{2}$ minutes after treatment with kinase and calcium. Yet, though it contained no antithrombin, it was not activated by calcium alone; the tube was watched for 38 minutes and then abandoned, as no sign of clotting had appeared.

EXPERIMENT 70.

This is quoted as an extreme instance of the variation in distribution of clotting-factors. The first experiment of the detailed series (No. 53) showed, in the case of an oxalated plasma, pure prothrombin in the watery, pure kinase in the chloroform-layer. In this instance, that of a plasma kept frozen for three days after bleeding, and then treated with chloroform for 1 hour at 37°, we find the exactly converse distribution. The finding of prothrombin and kinase, but no thrombin, although the plasma was not oxalated, is especially anomalous. We can only suggest that the precipitation of the

prothrombin with the curdy emulsion, which occurred in this instance owing to some unknown condition, protected it from activation by the liberated kinase, which, owing to some equally unknown condition, appeared in the watery-layer when separation was effected. Whatever be the cause, the watery-layer by itself had no effect on fibrinogen, and was equally inert after treatment with kinase and calcium; when, however, it was digested with prothrombin and calcium, the mixture produced clotting in 1½ minutes, showing an effective kinase. The chloroform-layer was inactive by itself, and did not activate prothrombin, but caused clotting in 3 minutes after it had been treated with kinase and calcium, showing that it contained prothrombin.

Hitherto we have quoted instances in which at least one of the layers, water or chloroform, obtained by the treatment described, contained one of the recognisable clotting factors, practically uncontaminated by others. We met with other cases in which the conditions were less simple. We have records of experiments, for example, in which both layers had some immediate thrombic action, but showed evidence of the presence, in addition, of both prothrombin and kinase, in that their thrombic action was increased by incubation with excess either of kinase or prothrombin, in presence of calcium. A puzzling feature in the behaviour of such samples was that simple incubation, with calcium only, did not greatly increase their content of thrombin; indeed, if incubation were protracted, their activity rather declined. This seems difficult to reconcile with the presence of prothrombin and kinase in them. We believe that the clue to this anomaly is furnished by the fact, which early came to our notice, that the preparations either of prothrombin or of thrombin, but more especially of the former, obtained by the chloroform method, are remarkably unstable; the kinase, by contrast, being remarkably stable under the same conditions. While the prothrombin or thrombin may show a very high potential or developed activity when first obtained, it shows a very rapid decline even at laboratory temperature, which decline is greatly accelerated by incubation at 37°. One experiment, which we have already quoted, showed, as the final stage of this degeneration, a preparation after two days' incubation, in which only kinase could be recognised. We must suppose then, that the solution in which thrombin, prothrombin and kinase can be detected, contains also some factor by which prothrombin and thrombin are continually being destroyed, the former with great rapidity; and that this action is greatly accelerated by incubation. The thrombic action detectable at any period can probably be increased, therefore, either

by accelerating the formation of thrombin, by adding extra kinase before the incubation, or by supplying excess of prothrombin, so that the formation of thrombin, by the kinase already present, does not fail through lack of the rapidly vanishing prothrombin. What, then, is the cause of this peculiar instability of prothrombin and thrombin thus prepared? A hint was afforded by the observation that many of the clots obtained by the use of these preparations, while hardening rapidly on formation, subsequently softened again, and might be completely dissolved in the course of a few hours' incubation. The solution was not a reversal of the clotting process; the mixture now yielded no further clot on the addition of thrombin, and gave no coagulum on heating to 60°. Clearly the initially formed fibrin had been digested. This might, perhaps, have been anticipated from Jobling's demonstration of the liberation of a tryptic ferment in serum shaken with chloroform—an action which we have repeatedly confirmed. And this tryptic action, ensuing on the destruction of the antitrypsin by the action of chloroform, explained not only the instability of the thrombin or prothrombin obtained by this method; it furnished also a possible clue to the appearance of kinase, the resulting activation of the prothrombin, and the whole series of phenomena under discussion. We proceeded to put this possibility to the test, by treating fowl's plasma with trypsin.

Effect of Trypsin on Fowl-plasma.

The trypsin used was the dried preparation of Fairchild, and this was made up as a 1 % solution in water as required. The sample of fowl-plasma used was that employed in Experiment No. 62. This plasma, at the time of the experiment with trypsin, had been kept in the cold-room for a week.

A few preliminary experiments showed that the trypsin itself had no coagulant effect on fibrinogen, and no activating effect on prothrombin in the presence of calcium ions. It contained, therefore, neither thrombin nor kinase.

Like all specimens of natural plasma or serum, the fowl-plasma possessed a marked antitryptic action, and it was necessary, in the first instance, to discover the amount of trypsin required to overcome this effect. It was found that five drops of the 1 % trypsin solution, added to 10 cc. of the plasma, had no apparent effect on the clotting properties during incubation for 1 hour. At the end of that period, a sample of the mixture was as free from activating effect, when added to prothrombin with calcium, as the

original plasma was. When the proportion of trypsin was increased ten times, however, the effect was very soon apparent.

EXPERIMENT 80 (*b*).

To 1 cc. of fowl-plasma were added 5 drops of 1 % trypsin, and the mixture placed in the water-bath at 37°. Samples were removed at intervals, and tested according to the usual scheme for kinase, as follows:

Prothrombin 5 + sample 2 + calcium 1, incubated 10 minutes; then added:

Fibrinogen 5 + saline 5.

The following results were obtained:

With original fowl-plasma.—No clot after several hours.

With sample after 3 minutes' tryptic digestion.—Hard clot in 5 minutes.

With sample after 6 minutes' tryptic digestion.—Hard clot in 2 minutes.

With sample after 15 minutes' tryptic digestion.—Hard clot in 1½ minutes.

There is clear evidence, therefore, of the production of kinase by the action of trypsin on the plasma. The question at once arises, however, why the formation of the kinase does not result in the clotting of the plasma itself, since the latter contains fibrinogen, prothrombin and calcium. As a matter of fact, the plasma does clot eventually. In the experiment above recorded, the residue of the plasma-trypsin mixture, left in the water-bath, set to a soft clot 5 minutes after removal of the last sample, and therefore 20 minutes after the commencement of the incubation. This clot did not harden, but, on the contrary, soon showed signs of dissolution, and had nearly disappeared at the end of 3 hours. Evidently we were dealing with a complex action of the trypsin, which throws considerable light on the anomalies encountered in dealing with the action of chloroform. The trypsin not only sets free kinase, but simultaneously digests prothrombin, thrombin, fibrinogen, and, later, the fibrin, if any should be formed.

Since several authors have shown that the temperature-coefficient of the action of thrombin above 17° is very small, while that of trypsin is much larger, it occurred to us that the excess of thrombin formed over that destroyed, at any period of the digestion, might be demonstrated by removing samples from the warm bath, and allowing them to cool to the room temperature. This expectation was verified as the following record shows.

EXPERIMENT 80 (*a*).

5 cc. of fowl-plasma + 25 drops of 1 % trypsin, were placed in the bath at 37°, and samples of 0·5 cc. removed, at intervals, to separate tubes, and

allowed to cool. The times indicated are the intervals after removal from the bath at which clotting occurred.

Sample removed immediately.—Did not clot in 3 hours.

Sample removed after 3 minutes' incubation.—Did not clot in 3 hours.

Sample removed after 6 minutes' incubation.—Clotted hard in 22 minutes.

Sample removed after 15 minutes' incubation.—Clotted hard in 5 minutes.

The remainder clotted softly after 20 minutes, without removal from the warm bath, as described above.

In another experiment, using only one-half the proportion of trypsin giving the above results, we detected no production of kinase or other change in the plasma after 30 minutes' incubation. The proportion of trypsin was then increased to that used above (5 drops of 1 % solution to each cc. of plasma) and the formation of kinase, with clotting of the plasma on cooling to room-temperature, occurred as before.

In another experiment the plasma was oxalated, and 1 % trypsin added in the proportion of 5 drops to 2 cc. of plasma. Again nothing occurred after 30 minutes' incubation, and again, after adding a further equal proportion of trypsin, so as to bring the total up to that which proved active with unoxalated plasma, kinase was formed after a further 10 minutes' incubation, as the following test shows (Exp. 84):

Prothrombin 5 + sample 2 + calcium 1, incubated 10 minutes; then added:

Fibrinogen 5 + saline 5.—Clotted hard in 4 minutes.

Naturally, in the absence of calcium ions, no clotting of the plasma occurred on cooling. Nor could any thrombin be detected by adding four drops to the usual fibrinogen-dilution.

It is quite evident that the results of treating fowl-plasma with trypsin are very similar to those obtained with chloroform, as described in the foregoing section. In both instances we have the liberation of kinase, which, if calcium is present, acts on the prothrombin of the plasma to form thrombin. With the knowledge that one effect of chloroform on plasma is to liberate trypsin, it seems justifiable to regard this as the primary action, to which the appearance of kinase and thrombin are secondary. It then becomes easy to understand why the effect of chloroform, on one batch of plasma, should vary with the time which elapses between the preparation of the plasma and the performance of the experiment. The fact, for example, that a plasma which, when fresh, readily gives a powerful thrombin on treatment with chloroform, may, after cold storage for a week or two, give only prothrombin, readily finds its explanation. In both instances the chloroform treatment

removes the antithrombic and antitryptic actions of the plasma. In the fresh plasma, active trypsin is thereby unmasked, with a resultant production of kinase and, unless calcium has been removed, of thrombin; in the older plasma, we must suppose that the trypsin has undergone decay, so that the only perceptible action of the chloroform is to remove antithrombin, and precipitate fibrinogen, leaving a solution in which prothrombin is the only clotting-factor which can be detected.

The supposed Antagonism between Kinase and Antithrombin.

Howell's [1911] conception of the function of kinase (thromboplastin) starts from the assumption that the substance, or substances, in plasma, which hinder the clotting action of formed thrombin, also hinder the activation of prothrombin by calcium. As we have already indicated, we can find no ground for this assumption, though it is peculiarly difficult to disprove. For the only test of the activation of prothrombin, namely, the appearance of thrombic activity, cannot be applied in the presence of substances by which that activity is inhibited. Howell, however, who considers the function of kinase (thromboplastin) to be the removal of antithrombin, permitting the activation of prothrombin by calcium, supports his conception by evidence designed to show that kinase neutralises the inhibitory action of normal or peptone-plasma, and of hirudin, on the action of formed thrombin.

It will be clear that, if evidence of this effect is to be unequivocal, it must be obtained under conditions which allow the balance between thrombin and antithrombin to be upset in only one way, namely, by the neutralisation of the antithrombin. If there is any possibility of an increase in the amount of thrombin present, the experiment appears to us of no value for the purpose. This criticism would exclude a considerable part of Howell's evidence on this point, in that the experiments were made with oxalated plasma, with the addition of calcium salts. According to Howell, "the acceleration in the time of clotting caused by the thromboplastin cannot be explained by assuming that it served to activate the prothrombin in the oxalated plasma, since the control experiment with calcium chloride showed that this substance alone was sufficient for the purpose." The control experiment in question showed, indeed, that, when calcium chloride was added to the oxalated plasma, sufficient thrombin was formed to clot the plasma in nine minutes. But it surely furnished no proof that the whole of the prothrombin was activated in that time, and that the addition of tissue extract would cause no increase

in the amount of thrombin formed. There is at least a possibility here of the balance between thrombin and antithrombin being upset by addition of thrombin, rather than removal of antithrombin.

The same criticism applies with even greater force to the experiments illustrating the activation of "a preparation containing both prothrombin and antithrombin" by tissue extract. To assume that the action, in this case, is a removal of antithrombin, would be to beg the whole question at issue.

If these experiments are excluded, as giving results which are explicable, with at least equal ease, on the Morawitz theory, there is left, of Howell's evidence, only the series in which fibrinogen solution, or oxalated and dialysed plasma were used, without addition of calcium. In this series the "antithrombin" used was not heated plasma, but hirudin. And the nett result of the experiments is to show that, while a proportion of hirudin, which neutralises the action of thrombin indefinitely, generally does so still, if tissue extract be added as well, smaller proportions, which greatly delay the action of thrombin, generally delay it to a smaller extent in the presence of tissue extract. The degree, in which the tissue extract weakens the hirudin action, may be gathered from the clotting times of parallel experiments.

Hirudin	With tissue extract	Without
1 drop	Less than an hour	2 hours 15 minutes
2 drops	3 hours	6½ hours (weak)
3 „	No clot	No clot
2 „	30 minutes	2–3 hours (imperfect)
4 „	2 hours 15 minutes	No clot in 24 hours

There are other experiments in which the difference produced by "thromboplastin" is much less than this. But, in the best case, what does it amount to? The effect of hirudin, sufficient to prolong clotting beyond 24 hours—say, for practical purposes, indefinitely—is so weakened, that clotting occurs after little more than two hours. But the effect, which the action thus demonstrated is supposed to explain, is something of a different order altogether. A fowl-plasma, for example, which remains stable for weeks or months, clots in a few minutes if some kinase be added. When, further, the notorious instability of hirudin is remembered, together with the presence of proteolytic ferments in tissue extracts, it becomes clear that there are several possibilities needing exclusion, before evidence like the above can be accepted as showing any genuine antagonism between thrombokinase and the antithrombin of plasma.

In our own experiments, made to test the existence of such an antagonism, we attached great importance to the exclusion of prothrombin from all the reagents used. We are testing the resultant action of a mixture of thrombin and antithrombin. If any prothrombin is present, and calcium not excluded, the addition of kinase will increase the balance in favour of thrombin. We may describe in detail an experiment in which these precautions were observed.

Our fibrinogen was a sample which had been rigidly tested in this direction, and found to be indefinitely stable with calcium and kinase. The antithrombin, obtained by heating fowl-plasma to 60° for 20 minutes, and filtering from coagulated fibrinogen, was subjected to a thorough test. Four tubes were put up, each containing 5 drops of fibrinogen solution, 2 drops of kinase, and 1 drop of calcium chloride (1 %). To these we added 1, 2, 3 and 4 drops respectively of the heated fowl-plasma. There was no sign of clotting in any tube after 24 hours. Therefore, so far as traces of prothrombin can be detected in the presence of antithrombin, we may conclude that the sample was prothrombin free.

The thrombin was obtained by shaking fresh fowl-plasma with chloroform and digesting for 1 hour at 37°. It was an extremely active specimen, and needed diluting many times to render its clotting-time susceptible of accurate measurement. After diluting 10 times, 1 drop clotted the usual fibrinogen dilution in 20 minutes. Two tubes were put up with this amount of thrombin and fibrinogen; to each was added one drop of calcium chloride, and to one of them 2 drops of kinase. Both clotted simultaneously. We may conclude, therefore, that the thrombin contained no remnant of prothrombin. That it contained traces of kinase is probable; but that would not affect the validity of the experiment.

After a few preliminary tests, to get a suitable mixture of thrombin and antithrombin, we put the following mixture into a tube and placed in the bath at 37°:

Fibrinogen 6 + antithrombin 15 + 4 times diluted thrombin 1 + calcium 1. After 10 minutes' incubation we divided the solution into two equal portions, added 2 drops of kinase to one, and returned both to the water-bath. It is peculiarly difficult to time accurately the beginning of the clotting-process in the presence of antithrombin, which not only delays the onset of the process, but also makes the hardening of the clot much slower. No difference, however, could be detected between the two tubes in this experiment. In both, the first signs of increasing viscosity occurred about 20 minutes from the first mixing, and the clots in both tubes were equally firm at 25 minutes.

A similar experiment was made, increasing the antithrombin to 20 drops, with 1 drop of diluted thrombin as before. After 5 minutes' incubation the mixture was divided again, 2 drops of kinase being added to one-half, and 2 drops of 30 % glycerol to the other. In each tube the first thickening began after about 40 minutes, and the clot became firm in about 50 minutes. Several similar experiments were performed, using fibrin-extract as thrombin, and heated rabbit or guinea-pig serum as antithrombin. In no case could we detect any definite acceleration of the clotting process by kinase. Yet the proportions of kinase were such as, when added to indefinitely stable fowl-plasma, produced clotting in a few minutes.

We are compelled, therefore, on this point also, to range ourselves with the earlier observers (Morawitz, Fuld and Spiro), and in opposition to Howell, in our inability to detect any real antagonism between kinase (thromboplastin) and antithrombin.

Howell's Prothrombin.

One of the greatest difficulties of any discussion on blood-clotting is the use of the current nomenclatures without prejudice to the point in dispute. In accordance with the original employment of the term by Schmidt, we have used "prothrombin" to denote the inactive precursor of thrombin, which is activated by kinase in the presence of calcium. Howell regards a preparation showing these properties as a complex of the thrombin-precursor with antithrombin. It is necessary to remember that, having supported this view of the constitution of prothrombin, by evidence which we have already discussed, Howell thereafter applies the name "prothrombin" to preparations which he regards as free from antithrombin, since they need only calcium for their activation. Such a preparation he obtained from cat-plasma by precipitating with acetone, washing the precipitate with ether, and drying in a current of warm air. Though we have followed Howell's directions as closely as possible, we have hitherto failed to obtain from rabbit-plasma a preparation yielding thrombin on treatment with calcium alone, though our preparation was readily activated when treated with kinase in addition. We found no evidence of antithrombic action after the preparation had been heated to 60° for 4 minutes. We hope to be able to obtain a preparation corresponding in properties to Howell's, and propose, in that event, to examine it for the presence of kinase by use of the fibrinogen and prothrombin preparations which we have employed in our other experiments. It is clear that the

value of the evidence, as to the properties of such a preparation, is dependent on the proved freedom from prothrombin of the fibrinogen used. If the test-fibrinogen were contaminated with prothrombin (in the original sense), it is evident that a solution containing only kinase would exhibit the properties of Howell's "prothrombin." And on this particular point, of the freedom of his fibrinogen from prothrombin, Howell's published evidence seems to us to be incomplete. His fibrinogen is described as being prepared by precipitation and reprecipitation from oxalated cat-plasma, with an equal volume of saturated sodium chloride. Rettger's precaution of clarifying the solution, by forming a filmy precipitate of barium phosphate—which possibly would produce the same effect as the barium sulphate used by us—was found unnecessary, since, without it, solutions were obtained which did not clot spontaneously on adding calcium. There is no evidence that they were tested with kinase in addition. This omission seems to us to weaken all the evidence obtained with such preparations; for to assume that prothrombin, if present, would be activated by calcium only, is to assume the point which the solution is used to demonstrate at a later stage of the investigation.

Summary and Discussion.

The following are the main experimental results of this preliminary investigation.

1. Indications have been obtained for the preparation of a set of test-solutions of the different factors concerned in the coagulation of blood-plasma. In particular it has been found possible to prepare

(*a*) Fibrinogen, free from perceptible traces of either prothrombin or kinase, from oxalated mammalian plasma, by adsorbing prothrombin with barium sulphate, and then precipitating the fibrinogen by half-saturation with sodium chloride.

(*b*) Prothrombin, free from perceptible traces of thrombin or antithrombin, by a modification of Mellanby's method.

2. Fresh fowl's plasma, when shaken with chloroform, readily yields a powerful thrombin. If the plasma be previously freed from calcium, prothrombin and kinase are formed by the same treatment, and can often be obtained apart by centrifuging the chloroform from the watery residue. If the treatment with chloroform be continued for some days at 37°, kinase is the only clotting-factor remaining.

3. A similar formation of kinase and (in the presence of calcium) thrombin occurs, when fowl's plasma is treated with an appropriate proportion of trypsin. The effect of chloroform is probably due, therefore, to the liberation of the normal tryptic ferment of the plasma, by the destruction of antitrypsin.

4. We have failed to confirm the antagonistic action of kinase on antithrombin, described by Howell.

So far as these results have an incidental bearing on theories of blood-coagulation, they seem to us to be definitely in favour of the necessity of three factors for the formation of thrombin—prothrombin, kinase and calcium. They are not compatible with the more recent conception of Howell, according to which the function of the kinase is to inactivate the antithrombin, which prevents the activation of prothrombin by calcium.

We may turn from the results of experiment, and conclusions directly deducible therefrom, to some considerations of a more speculative nature.

For the more direct purposes of our investigation, the point of most significance, and of most novel interest, is the demonstration of the appearance of kinase, as the result of slight tryptic digestion, in a body-fluid which previously contained none. It seems possible that this phenomenon will prove to be of considerable physiological interest. Hints of such fermentative production of kinase can be found in earlier observations—for example, in Howell's statement that a tissue yields a more powerfully kinasic extract, if it be allowed to undergo some degree of autolysis at room temperature before extraction. It may be that some degree of protein cleavage is the necessary preliminary to the liberation of kinase from any tissue; and that the ease with which it appears on cell-injury, and the relatively drastic treatment needed for its production in a stable avian plasma, represent a differing delicacy in the poise of the balance between proteolytic ferment and those antagonistic influences which, in the case of the plasma, we summarily describe as "antitrypsin."

Much further experiment is needed to define the relation between this liberation of thrombokinase, and the toxicity acquired by serum and other native proteins, when subjected to a similar partial hydrolysis, and speculation on the basis of existing results would be premature. We have, as yet, no evidence as to which constituent of the plasma is involved in the hydrolytic production of kinase, though evidence, on the one hand, as to association of kinasic action with a lecithin-like substance (according to Howell with the

kephalin-fraction of tissue-lipoid), and, on the other hand, as to the presence of such a constituent in the euglobulins, must be regarded as suggestive. To this suggestion, and to the relation between kinase-formation and the toxicity of variously treated sera and proteins, including the specific toxicity of foreign protein to the anaphylactic animal, we hope to return in future communications.

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XXX. CREATINURIA IN CHILDREN.

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(*Received July 27th, 1916.*)

It has been known for some years that creatine is constantly found in the urine of young children although none appears in normal adult urine, or at any rate in that of male adults. As yet nothing appears to be known concerning the cause of this difference.

Rose [1911], who examined specimens from a number of children and young people, found that creatine was usually present until the age of puberty. His observations were not made on 24-hour specimens of urine, and in many cases the diet contained meat. Folin and Denis [1912, 1] also made observations on a number of normal healthy children, and found that the creatine formed a fairly large fraction of the "total creatinine" excreted, even in the case of vegetarians. It was also shown that the ingestion of meat was followed by a rapid rise in the creatine output. They made a few observations on morning, afternoon and night samples, but these did not lead them to any conclusions concerning the relative amounts of creatine excreted during different parts of the day. Krause [1913] has also made observations on the creatine excretion of children, and concludes that creatine occurs in the urine of normal boys up to about six years of age, and for a longer time in the case of girls. To determine the fate of ingested creatine he gave two boys 0·3 g. of creatine in the morning. One, aged eight, who was previously excreting no creatine, excreted 43 % of that given, and the other, aged five, excreted at least 22 % of that given (one sample of urine being lost). Approximately the same amount was given to two girls the last thing at night; one, aged eleven, previously excreting no creatine, excreted during the next 24 hours 31 % of that given, and the other, aged six, excreted 56 %. In no case was the ingestion of the creatine followed by any apparent increase in the excretion of creatinine.

According to some observers, e.g. Plimmer, Dick and Lieb [1909], adults can take as much as 2 g. of creatine per day without any of it appearing in the urine, as such or as creatinine, though the more recent results of Myers and Fine [1915] show some excretion by one subject when only 1 g. was taken, and there was apparently a slight conversion of creatine to creatinine by both subjects when larger amounts of creatine were taken.

The starting point of our own experiments was a case of amyotonia congenita on which we were making a fairly detailed metabolism study. The urine was found to contain a very large amount of creatine relative to the creatinine, and in order to study the relationship more closely the separate samples of urine passed during the 24 hours were examined. For comparison a similar study was then made on a normal boy.

The determinations of both creatinine and creatine were made essentially by the new Folin [1914, 1] method. One or two points with respect to this method noticed during our investigation may be mentioned here. When the urine is so dilute that more than 5 cc. are required for 1 mgm. of creatinine, Folin recommends diluting the standard also before adding the alkali. If, however, the urine is so dilute as to require 15 cc., a case we sometimes encountered (after making to a suitable volume), this method is not very convenient if a number of observations have to be made on one day; for under these conditions the colour is at first about 15 % less than if the same amount had been present in 1 cc., but increases on standing, so that after 3 hours the readings would be much more nearly equal. We therefore diluted the standard creatinine solution to 5, 8, 12 and 15 cc., and compared the colorimeter readings at between 3 and 9 minutes after making to volume with that of the standard where no water had been added, this being set at 1·00 cm. The readings were 1·015, 1·02, 1·06 and 1·19 respectively. With these figures we were then able to correct the readings when using any volume of urine up to 15 cc., without requiring to make a new standard for each determination.

McCrudden and Sargent [1916] have recently thrown doubt on the accuracy of the new Folin method of estimating creatine in urine, since they find that this method gives higher results than the hydrochloric acid conversion, and gives some "apparent creatine" in almost every urine, though all the figures quoted seem to be from nearly normal patients. Our experience does not agree with that of McCrudden and Sargent. With a sample of normal adult urine a reading of 0·99 cm. (mean of four settings of colorimeter) was found for creatinine; exactly the same reading was found after treatment for

conversion of creatine by the new Folin method (except that broken glass was substituted for pebbles), whereas the hydrochloric acid method gave a reading of 1·01, i.e. apparently a slight loss, but within the limits of error. We have nearly always used a few small pieces of porous pot to facilitate steady boiling. A disadvantage of this is that after adding the alkali a rather gelatinous precipitate is formed. But if after making to volume one allows the mixture to stand for 10 minutes, this settles sufficiently to enable enough clear solution to be pipetted from the top. If one prefers to centrifuge, the solution used for the estimation of the preformed creatinine should also be centrifuged, as the colorimeter reading is about 3 % higher after centrifuging, which, though almost negligible for the preformed creatinine, may considerably influence the creatine figure.

In the creatine estimation we have always made the volume to 20·5–21·5 cc. before adding the alkali in order to avoid any errors caused by differences in dilution, since the correction for dilution used for creatinine is not suitable for creatine if one desires to wait for the precipitate to settle. For most of the determinations in urine for which McCrudden and Sargent give details the volume of urine and picric acid solution was 23 cc. in the creatinine determination, 20 cc. in that of creatine by the new Folin method, and at least 25 cc. where hydrochloric acid was used. These differences in dilution are in the order which, according to the results given above, would give an "apparent creatine" value, but we should expect any error due to this to be not greater than 2 % of the creatinine value, whereas in many cases their "apparent creatine" is much higher than this.

In our further determinations to test the method of creatine estimation, a sample of normal adult urine, for which when using 1 cc. the creatinine reading was 0·58 when centrifuged (0·555 not centrifuged), read also 0·58 when centrifuged after treating by the new Folin method for creatine, using porous pot. A sample from another normal adult read 1·11 for creatinine (1·085 when not centrifuged) and for creatine 1·105 when boiled for 2 hours, and 1·11 when boiled for 5 hours, and both centrifuged. It is seen that our results with three different samples of adult urine, all quite normal so far as known, do not agree with McCrudden and Sargent's, which, at any rate for the most part, were obtained on nearly normal urine; and if it is granted that normal male adult urine contains no creatine, our results are quite satisfactory.

In connection with the creatine estimation we noticed that after the urine had been boiled with picric acid, the development of the colour on adding the

alkali appeared to proceed much more rapidly than when the mixture had not been boiled. This was not due simply to a difference in temperature, since we usually allowed the solution used for creatine estimation to stand overnight after boiling and making to 21 cc., the creatinine determination being made at the same time.

The volumes of urine taken for the estimation of the creatinine and creatine in any one sample were always such that the readings were very nearly the same, so as to avoid any error due to the reading not being exactly proportional to the amount present. Throughout the paper and on the curves the creatine values are all given as their equivalent of creatinine.

For convenience the results with the normal boy will be dealt with before those from the amyotonia case. The child investigated was three years old and the diet was free from meat and meat extract. Meals were taken each day at about 8 a.m., 1 p.m. and 6 p.m. In the first period the collection extended over four days (Jan. 4–8) and in the second over eight days (Feb. 27–Mar. 5). To give the results for all the samples passed during these periods would occupy too much space, and on account of the bladder not being emptied at the same time each day it is almost impossible to give an average figure for the different parts of the day. Curves showing the amounts of creatinine, creatine and total nitrogen excreted per hour during the 4-day period, as well as the percentage of "total creatinine" excreted as creatine, are given in Fig. 1[1]. The most significant fact shown by these curves is the very small excretion of creatine during the night, whether this is measured in absolute amount or as percentage of the "total creatinine." It will also be observed that, measured in either way, the amount is larger on the whole for the morning than the afternoon.

The excretion of creatinine per hour during the night is also seen to be lower than the average during the day-time, but only slightly so. The curve for total nitrogen is similar in character to those for creatine and creatinine, showing less variations between day and night than the creatine but greater than the creatinine. The curve representing volume per hour (not inserted in Fig. 1) runs fairly parallel to that for creatine, though exceptions have been observed both in this and in subsequent periods.

The child slept soundly from a short time after the beginning of the night

[1] The daily creatinine values for this period average 254 mgm. In two succeeding periods they were found to be 231 and 239 mgm. The improbability of this variation led to an examination of the standard creatinine solution being made, and it was found to have undergone some decomposition. The values for creatine and creatinine in this period are therefore probably 7 % too high.

period to within a short time of its close. It may be pointed out that the amount of creatine excreted during the whole of the night period averages only 7 mgm., an amount approximately equal to that during the last 1½ hours before bed or the first hour during the morning. Consequently a large part of that found during the night period may have been excreted before going to sleep or after waking, and not during the actual sleep period. On the first and third days he also slept for about 2 hours during the afternoon, but this does not seem to have affected the results appreciably.

The results obtained during the 8-day period were quite in agreement with those given in Fig. 1, except that on only one day was the creatine output during the last period before going to bed greater than during the

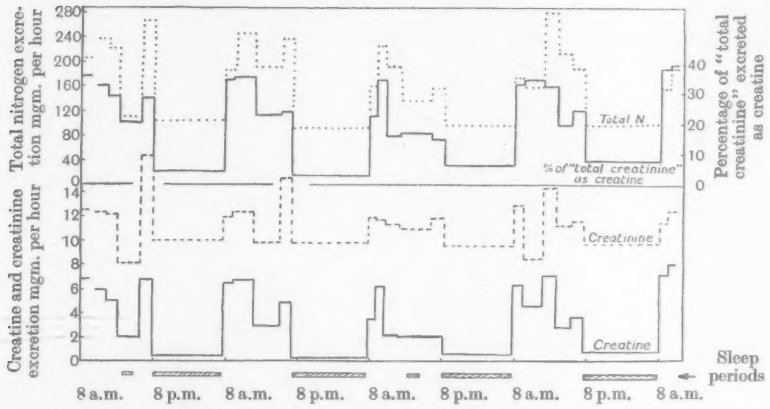

Fig. 1.

preceding period. At 8 p.m. on the third day 120 cc. water were given, and at the same time on successive days, 130 cc. milk, 16 mgm. creatine (= 13·8 mgm. creatinine) in 40 cc. water, and 31·4 mgm. creatine (= 27·1 mgm. creatinine) in 50 cc. water, but apparently none of these had any influence on either the creatine, creatinine, total nitrogen, or volume excreted, these all being normal as judged by the two preceding and succeeding nights and days.

It was then recognised that although the child may be able to deal with an appreciable quantity of creatine when given at the beginning of the night period without any of it being excreted, yet this may not be so when it is given at the beginning of the day period, since during this time a relatively large amount of the total creatinine is excreted as creatine, indicating either

a greater rate of production or smaller rate of utilisation, or both, compared with the night period. The following experiment shows fairly conclusively that the difference in the power of utilisation is an important factor. Observations were carried out for six days, from 7 a.m. on April 3rd to 7 a.m. on April 9th, and the urine for each day was collected in three periods, 7 a.m to 1 p.m., 1 p.m. to 7 p.m., and 7 p.m. to 7 a.m., the bladder being emptied at a time very near the end of each period. On April 7th it was noticed that the child had developed a slight cold, but there was no fever. At 7 o'clock on the *evening* of the third day 102 mgm. of creatine (= 88 mgm.

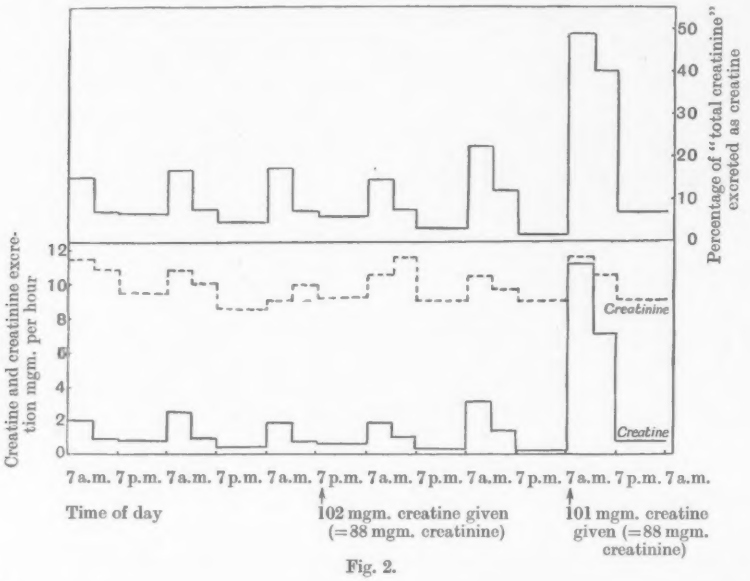

Fig. 2.

creatinine) were given by mouth in 30 cc. water, and at 7 on the *morning* of the sixth day this dose was repeated. The curves in Fig. 2 show the rate of creatinine and creatine excretion, in mgm. per hour, during the six days, as well as the percentage of the "total creatinine" present as creatine. From the curves it is easily seen that the creatine when given in the evening has no appreciable influence on the excretion of either creatine or creatinine, but when given in the morning there is considerable increase in the creatine excretion during the next 12 hours, but none in the creatinine excretion. The following Tables (I and II) emphasise the points just mentioned, the

second and third lines giving the rate of excretion up to 24 hours after taking the creatine, and the first line the average values for the rest of the period.

TABLE I.

Creatine excretion. Mgm. per hour.

	7 a.m. to 1 p.m.	1 p.m. to 7 p.m.	7 p.m. to 7 a.m.
Average for normal days ...	2·3	0·94	0·43
Creatine (=88 mgm. creatinine) given at 7 p.m.	1·8	0·95	0·59
Creatine (=88 mgm. creatinine) given at 7 a.m.	11·1	7·0	0·68

TABLE II.

Creatinine excretion. Mgm. per hour.

	7 a.m. to 1 p.m.	1 p.m. to 7 p.m.	7 p.m. to 7 a.m
Average for normal days ...	10·4	10·1	9·0
Creatine (=88 mgm. creatinine) given at 7 p.m.	10·5	11·5	9·15
Creatine (=88 mgm. creatinine) given at 7 a.m.	11·5	10·5	9·05

The figures for each part of the 24 hours after taking creatine are all within the limits of variation for normal days except those for the morning and the afternoon period after its administration at 7 a.m.; during these the excess above the normal excretion was 50 mgm. from 7 a.m. to 1 p.m. and 37 mgm. from 1 p.m. to 7 p.m., giving a total of 87 mgm., that is, equal to the amount given.

It was noticed that the urine for the morning period beginning 24 hours after the last administration of creatine contained an appreciable amount of acetone and a trace of aceto-acetic acid, and the creatine content was higher than normal. Acetone had never been found in any of the previous samples of urine from this child. In order to be quite certain that this high creatine excretion was an adventitious circumstance associated with the slight acidosis, the urine was again collected for 4 days beginning 7.30 a.m. April 18th. At 7.30 on the morning of the third day, 99 mgm. of creatine (= 86 mgm. creatinine) dissolved in 30 cc. water were given. As in the preceding period the creatine excretion was greatly increased during the ensuing morning and afternoon as shown by the results in Table III.

TABLE III.

Excretion of creatine. Mgm. per hour.

	7.30 a.m. to 1.30 p.m.	1.30 p.m. to 7.30 p.m.	7.30 p.m. to 7.30 a.m.
Average of 1st, 2nd and 4th days	6·5	2·7	0·90
3rd day	13·9	4·3	0·34

On this occasion there was no acetone in the urine on the morning of the fourth day, and the creatine was quite normal in amount, showing that the acetone and high creatine on the preceding occasion were not due to the creatine taken on the preceding morning.

The amount of creatine excreted during the third day in excess of the normal for this period was 45 mgm. from 7.30 a.m. to 1.30 p.m. and 10 mgm. from 1.30 p.m. to 7.30 p.m., giving a total of 55 mgm., each reckoned as creatinine. In this period we see that less than two-thirds of the creatine given in the morning was recovered in the urine, whereas in the preceding period apparently the whole amount was recovered.

In adults the excretion of creatine is usually associated with acidosis, in consequence of which a larger percentage of the total nitrogen is excreted as ammonia than by normal adults. In order to see how far similar conditions held in the child under investigation, the ammonia and the total nitrogen were estimated in each sample of urine during the last period, using the van Slyke and Cullen [1914] method for ammonia. Apparently the excretion of these two substances was not appreciably affected by the creatine administration.

The average results for the period, excluding the 24 hours after taking creatine, are given in Table IV.

TABLE IV.

	7.30 a.m. to 1.30 p.m.	1.30 p.m. to 7.30 p.m.	7.30 p.m. to 7.30 a.m.
Creatinine. Mgm. per hour	11·1	10·4	8·55
Total nitrogen. Mgm. per hour	184	143	86
N as ammonia. Mgm. per hour	5·43	3·26	5·27
Percentage of total N as NH_3	2·95	2·28	6·12
Percentage of "total creatinine" as creatine	37	20·6	9·5
Creatine. Mgm. per hour	6·5	2·7	0·90

It is seen that the percentage of the total nitrogen excreted as ammonia is highest at night when the creatine is lowest, and is slightly less during the morning than during the afternoon period, although the creatine is much greater.

In accordance with the ammonia results, the night urine was acid to litmus on each occasion, whereas that for the morning and afternoon was alkaline. The conditions then are quite different from those found to hold when creatine is excreted by adults. Moreover as pointed out by Folin and Denis [1912, 1], if the creatine excretion by children is due to lack of carbohydrate, one would expect it to be particularly high in the night urine, whereas our observations show that the reverse holds good.

Milk Factor. During and for one day preceding each period, the diet, as mentioned previously, was free from meat and meat extracts, and consisted chiefly of bread and butter, milk, porridge, milk pudding, vegetables, fruit, and eggs.

Folin [1914, 2], however, has found that milk contains an appreciable quantity of creatine. One sample examined by us, using the method advocated by Folin, contained 2 mgm. of creatinine and 9 mgm. of creatine (reckoned as creatinine) and another sample 2 mgm. creatinine and 8 mgm. creatine per 100 cc. Including the milk pudding and porridge, the child under examination took about 300 cc. of milk each day from the same source as the second sample, and containing therefore about 24 mgm. of creatine. Our experiments on the ingestion of creatine indicate that the fate of any creatine contained in the food will depend largely on the capability of the body for utilising or destroying it at the time that the creatine is being absorbed. This capability is best indicated by the smallness of the amount of endogenous creatine being produced at the same time. It is probable therefore that on a milk diet a portion of the creatine excreted during the day is of exogenous origin, but at night practically the whole of the exogenous creatine may be utilised.

We should like to emphasise the creatine content of milk, since it has usually been allowed when a diet free from creatine was desired.

The day and night urine for another normal boy aged $2\frac{1}{2}$ years was also examined, and here again it was found that the excretion of creatine, both in absolute amount and as percentage of the "total creatinine," was less during the night than during the day. But the difference between the night and day periods was much smaller than in the preceding case, probably because this second boy awoke once or twice during the night and received 180 cc. of milk just before going to bed and another 180 cc. during the night.

The case of amyotonia congenita with which we were concerned was a girl four years old, on whom a fairly detailed metabolic study was carried out, the results of which are being published elsewhere [Powis and Raper, 1916]. In this case the daily urine contained about 67 mgm. creatinine but about 118 mgm. creatine (reckoned as creatinine), so that about 64 % of the "total creatinine" was present as creatine. As in the preceding cases, during the experiment and for 43 hours before its commencement, the diet was free from meat and meat extract, but it contained about 800 cc. of milk per day. The results on two normal days are shown by the first portion of the curves in Fig. 3. On these days the rate of creatine excretion is practically

the same during the morning and afternoon, but is less during the night. The percentage of "total creatinine" as creatine is less during the afternoon than the morning, and still less at night. The creatinine excretion, judged by these and the two succeeding days, shows no definite variation with the time of the day.

It is seen that the variations in the creatine excretion during the different parts of the day are similar in character to those recorded above for the normal boy, but are much less marked, possibly because this subject spent the whole of the day in bed. The child went to sleep and awoke earlier than the normal boy and in accordance with this it will be noticed that the greater rate of creatine excretion also starts at an earlier hour, but as with the normal boy, occurs in the first period after sleep.

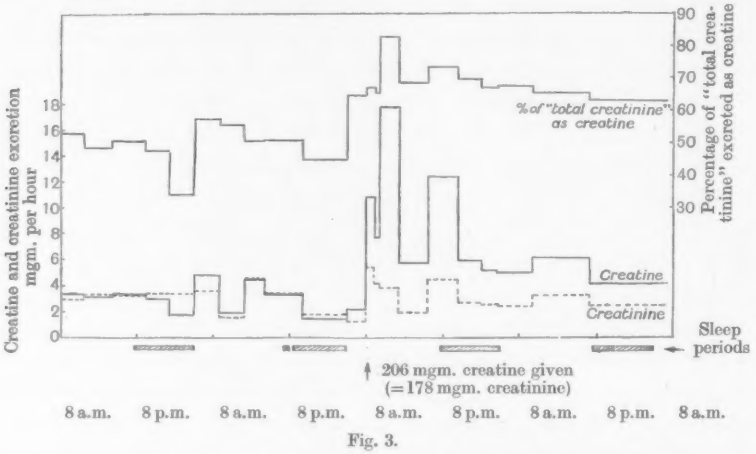

Fig. 3.

At 8 a.m. at the beginning of the third day 206 mgm. of creatine (= 178 mgm. creatinine) dissolved in 20 cc. of water were given. There was immediately a marked rise in the rate of creatine excretion and the rate continued to be higher than normal throughout the ensuing 48 hours; this is true even for the two night periods, whereas with the normal boy the administration of 102 mgm. of creatine at either 7 a.m. or 7 p.m. did not influence the creatine excretion during the night period. This finding is quite in accordance with the high creatine excretion of this subject, and shows a smaller capacity for utilising or destroying this substance. The total amount of creatine excreted during the 48 hours following its administration was 311 mgm., whereas during the preceding 48 hours 138 mgm. were excreted.

This gives an excess of 173 mgm., which is almost equal to the amount taken (178 mgm.). There is no increase in the creatinine excretion, the total amount during the preceding 48 hours being 137 mgm. and during the succeeding 48 hours 130 mgm.

Acetone was occasionally present in the urine of this subject, but there was none in any of the samples during this period of observation.

Our results on the ingestion of creatine by children are not incompatible with those of Krause, who did not compare the night and morning administration on the same case, and gave a larger amount, viz. 0·3 g.

Table V contains the data for the average amounts excreted per 24 hours on the days when no creatine was given, as well as the amounts per kilo. of body weight.

TABLE V.

Subject	Age (years)	Weight (kilogram)	Total N. (g.)	Creatinine (mgm.)	Creatine (mgm.)	% of total creatinine as creatine	Mgm. creatinine per kg.	Mgm. creatine per kg.
Normal boy F.P.R.								
Apr. 3rd to 9th	3	14	—	231	25	9·6	16·5	1·8
Apr. 18th to 22nd	3	14	3·10	239	59	20	17·1	4·2
Normal boy G.B.L.	2½	14	4·34	192	81	30	14	5·8
Amyotonia, Girl (average of three periods—in all 13 days)	4	9·7	3·73	67	118	64	6·9	12·1

The two other periods with F.P.R. gave 18·3 and 17·6 % of the "total creatinine" as creatine, but owing to some decomposition of the standard the exact amount of creatinine and creatine is not known. Assuming the creatinine to be 235 mgm. per day, the creatine in these periods was 57 and 50 mgm. per day. The figures for the normal children are in fair agreement with those found by Folin and Denis [1912, 1] and by Krause [1913] as far as comparison is possible.

The above results show the great difference between the amyotonia congenita case and normal children. Morse [1916] has recently published a statement that he has determined the creatinine and creatine in the urine of children with various diseases involving atrophy, and in all of them the creatine content was high.

It will also be noticed that the creatine excretion of the normal boy F.P.R. was much less during the period April 3rd to 9th than during the other periods. This is perhaps due to the fact that he had spent several weeks in the country just before this period.

It has been suggested that the non-appearance of creatine in the urine (either as such or as creatinine) when given to adults is due to its destruction in the intestine by bacteria [Twort and Mellanby, 1913]. Our experiments do not support this view. In the case of amyotonia congenita the increased excretion of creatine after its administration was apparent so early that we are forced to believe that the creatine was rapidly absorbed, which agrees with the experiments of Folin and Denis [1912, 2] on cats. Further, the administration to the boy F.P.R. in the morning took place about half an hour before breakfast when the intestines may be considered to have been empty and therefore bacterial action on the creatine favoured. In the evening it was administered about 1½ hours after the last meal and would therefore be passed into intestines containing food, conditions unfavourable to the bacterial degradation of creatine. Yet in the former case there was increased excretion, but not in the latter.

Discussion.

The results so far obtained with regard to creatinuria in children are most simply explained by assuming that this is due to the immaturity of the muscles. Creatine is not entirely an index of endogenous metabolism in the way that creatinine is, because the two do not show parallel variations. This may be due to the fact that creatine is utilisable, whereas creatinine is not. The production of creatine at night is probably lower than during the day, and anabolic processes of rest, by utilising or destroying part of it, cause a further diminution in the amount excreted. Sleep is apparently not the only factor involved, since the creatine excretion during the afternoon is less than during the morning, especially when reckoned as percentage of the "total creatinine." Assimilation of food has apparently little or no influence, since taking 130 cc. of milk at 8 p.m. did not influence the creatine excretion during the ensuing night. The chief factor appears to be the state of rest of the skeletal muscles associated with the temporary cessation of voluntary control which occurs during sleep.

The fully matured muscle produces only creatinine, and no creatine, or only such small amounts that utilisation processes prevent their coming to excretion. As the child develops, the creatinine coefficient gradually increases, and the creatine diminishes until it finally disappears. An extreme case of immaturity is seen in amyotonia congenita, in which the muscles are soft, toneless, and under-developed. Accompanying this condition, the

excretion of creatinine per kilo. body-weight was much lower, and that of creatine much higher, than for a normal child of the same age.

Summary.

(1) Suggestions are made improving the new Folin method of estimating creatinine in the case of urine very dilute in respect to this, and observations are recorded which support the accuracy of the new Folin method of creatine estimation.

(2) The diurnal and nocturnal variations in the excretion of creatinine and creatine have been determined for a normal boy, aged $2\frac{1}{2}$ to 3 years.

(3) The rate of creatinine excretion is slightly less during the night than during the day.

(4) The rate of creatine excretion is highest during the morning, less during the later part of the daytime, and is very small during the night.

(5) If 0·1 g. creatine is given at the beginning of the morning most of it is excreted as such in the urine during the ensuing 12 hours, but if the same amount is given just before going to bed, there is no increase in the creatine excretion.

(6) The percentage of the total nitrogen in the urine which is present as ammonia is smaller during the day than during the night, showing that the higher creatine excretion during the day is not due to greater acidosis.

We have much pleasure in acknowledging that the expenses of this investigation have been defrayed by the Medical Research Committee.

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XXXI. NOTE ON SOME EXPERIMENTS WITH GLYOXALINE DERIVATIVES.

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(*Received August 7th*, 1916.)

This note deals (1) with the behaviour of glyoxaline-formaldehyde in the organism of the dog, (2) with the condensation of glyoxaline-formaldehyde and malonic acid, (3) with the action of sulphuric acid on α-hydroxy-β-glyoxaline-propionic acid.

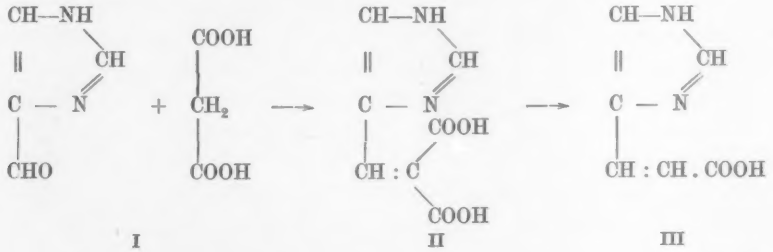
I. Furfuraldehyde, when injected hypodermically into the dog, was shown by Jaffé and Cohn [1887] to be changed principally into pyromucic acid and the condensation production of this acid with glycine, namely pyromycuric acid, but to the extent of a few per cent.; there is also produced furfurylacryluric acid, which is formed from glycine and furfurylacrylic acid. This latter observation induced us to administer glyoxaline-formaldehyde to a dog, in the hope that it might similarly be converted into urocanic acid, identified by Hunter [1912] as glyoxalineacrylic acid, which in two dogs has been observed as an abnormal, yet constant product of metabolism.

Accordingly 2 g. of glyoxaline-formaldehyde, for which we are indebted to the kindness of Dr F. L. Pyman [1916], was given hypodermically in 40 cc. of water to a small terrier bitch. The 280 cc. of urine, collected in the first 24 hours, was evaporated in vacuo and the residue was extracted with methyl alcohol, which removed the whole of the substances giving Pauly's reaction. After evaporation of the methyl alcohol, the residue was dissolved in 200 cc. of 5 % sulphuric acid and precipitated with 90 g. of phosphotungstic acid (a slight excess); the precipitate, filtered off next day, dissolved almost completely in 280 cc. of boiling water, and on cooling a large part remained in solution. From the phosphotungstate insoluble in cold water no base could be isolated, but the solution yielded glyoxalinecarboxylic acid, as follows. The bases, after recovery by means of barium hydroxide and

evaporation to dryness, were almost completely soluble in methyl alcohol, which left on evaporation a residue consisting largely of urea, but also giving a strong Pauly reaction. Most of the urea was removed by adding dilute nitric acid and the filtrate was neutralised and evaporated. The residue, dissolved in a little hot dilute alcohol, deposited a small quantity of a glyoxaline derivative which on recrystallisation from a mixture of methyl alcohol and acetone formed thin needles, closely resembling glyoxaline-carboxylic acid. A crystal of the acid obtained from the dog grew in a solution of the synthetic acid. The acid from the dog's urine melted at 272–273°, another specimen, recrystallised from water, at 279°, the synthetic acid at 275–276°, the mixture of the two at 276°. (The melting point depends greatly on the rate of heating; in the above cases the bath was previously heated to 260°.) The acid from the dog's urine on melting yielded a distillate which crystallised on cooling and then had the melting point 86–87°. (Glyoxaline melts at 88°.)

Micro-Kjeldahl. 0·0040 g. gave 4·95 cc. N/70 NH_3. N, 24·75 %. Calculated for $C_4H_4O_2N_2$: N, 25·0 %. This formation of glyoxalinecarboxylic acid from its aldehyde is therefore analogous to the production of pyromucic acid, observed by Jaffé and Cohn, but the yield was very small and no trace of urocanic acid could be detected, perhaps because the glyoxaline ring is broken down too readily by oxidation.

II. It was thought that urocanic acid might be formed *in vitro* from the aldehyde and malonic acid, with subsequent removal of carbon dioxide.



I II III

The substance II (*glyoxaline-methylidene-malonic acid*) is readily produced but so far it has been impossible to transform it into urocanic acid (III).

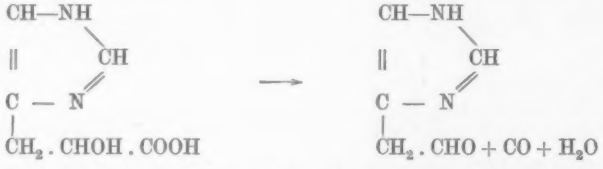
Knoevenagel [1904] showed that a variety of aldehydes readily condense with malonic acid and allied substances, when the reaction is catalysed by the addition of ammonia or a primary or secondary amine. The base is supposed to unite with the aldehyde giving a product which reacts readily

with the malonic acid. In the present case it is interesting to note that the basic glyoxaline-formaldehyde readily condenses with malonic acid without addition of a catalyst, on simply warming the two substances in aqueous solution on the water-bath. The aldehyde (1 g.), malonic acid (1·2 g.) and water (25 cc.) were heated together. A practically clear solution resulted from which almost at once the condensation product (1·6 g.) began to separate as a finely crystalline meal. The substance is sparingly soluble in water or acetic acid, and very slightly soluble in alcohol or ether. It crystallises from water in prisms, which melt at 214° with decomposition and evolution of much gas. It gives a finely crystalline sparingly soluble *nitrate*, thus resembling urocanic acid. The substance is strongly acidic and may be fairly accurately titrated with baryta and phenolphthalein, the acid neutralising two equivalents of alkali. Permanganate is readily decolorised in both alkaline and acid solution.

Micro-Kjeldahl. 0·0128 g. gave 9·65 cc. N/70 NH_3. N, 15·1 %. Calculated for $C_7H_6O_4N_2$: N, 15·4%.

All efforts to remove one molecule of carbon dioxide from the substance, so as to yield urocanic acid, were unsuccessful. The substance was heated directly with and without glycerol to 200°, with water in a sealed tube to 150°, with acids and with alkalies. Carbon dioxide was in many cases readily removed, but urocanic acid could not be isolated from the products of the reaction. With water at 150° the reaction product was very soluble in water and could not be crystallised.

III. With a view to obtaining glyoxaline-acetaldehyde in the same way that acetaldehyde is formed from lactic acid, α-hydroxy-β-glyoxaline-propionic acid was heated with sulphuric acid, and a molecular proportion of carbon monoxide was indeed eliminated. The primary reaction is no doubt expressed as follows:



but then the aldehyde undergoes the further loss of a molecule of water and two molecules are condensed, the whole reaction being represented as follows:

$$2C_6H_8O_3N_2 = C_{10}H_8N_4 + 2CO + 4H_2O.$$

The constitution of the base $C_{10}H_8N_4$, containing two glyoxaline rings, has

not yet been established, but it is evidently closely analogous to the hydrocarbon $C_{16}H_{12}$ obtained by Breuer and Zincke [1878] by heating phenylacetaldehyde and allied compounds with sulphuric acid, diluted with an equal volume of water. The hydroxy-glyoxaline-propionic acid was prepared from histidine by Fränkel's method [1903] and the substance containing a molecule of water of crystallisation was heated with three times its weight of sulphuric acid. With acid more dilute than 75 % no carbon monoxide is evolved until the acid has been concentrated. With 85 % acid evolution is slow at 150°, but complete at 170–180°. With 90 % acid carbon monoxide begins to appear at 125° and is liberated completely at 150–160°. With acid of a still higher concentration charring is apt to occur. The best concentration is therefore 85–90 % of H_2SO_4 and the yield of carbon monoxide approaches closely to the theoretical.

0·312 g. of hydrated α-hydroxy-β-glyoxaline-propionic acid (= 1/50 gram-molecule) was heated with 1 cc. of 85 % sulphuric acid in a test tube in a metal bath. Previous experiments had shown that no trace of carbon dioxide is formed in the reaction, so the air in the test tube was swept out by means of a current of carbon dioxide and subsequently the monoxide formed was collected in the same way in a Schiff nitrometer. The gas evolved measured 48·0 cc. at 20° and 760 mm., of this 0·2 cc. was absorbed by alkaline pyrogallol and 46·6 cc. by ammoniacal cuprous chloride, leaving a residue of 1·2 cc. The oxygen and the residue, probably nitrogen, were doubtless introduced as air from the Kipp apparatus.

46·6 cc. CO (moist) at 20° and 760 mm. = 0·052 g. or 93 % of the theoretical.

At the end of the experiment the sulphuric acid solution was pale brown and strongly fluorescent; the fluorescence is due to a substance hardly soluble in hot water, but readily in alcohol, which could not be obtained pure. The sulphuric acid may after dilution be neutralised with barium carbonate and after filtering hot, the base $C_{10}H_8N_4$ crystallises from the filtrate, or it may be obtained more simply by the addition of concentrated ammonium hydroxide, which at once precipitates it; yield 55–68 % of the theoretical. The deficiency is due to the formation of the fluorescent substance and of a substance readily soluble in water but not in alcohol, which can be obtained from the final mother liquor after quantitative removal of the sulphuric acid by barium hydroxide. This second substance is probably glyoxalinecarboxylic acid; it crystallised from water in thin needles, closely resembling the synthetic acid, and melted at the same temperature (284°).

Properties of the base $C_{10}H_8N_4$.

The base is readily soluble in cold alcohol, but only slightly in hot water (about 1 %), from which it is best recrystallised by addition of charcoal. In the crude state it forms small characteristic lozenge- or diamond-shaped plates, with rounded corners, but after recrystallisation irregular leaflets are obtained, which may be a centimetre long but still tenaciously retain a little colouring matter; by sublimation in the vacuum of a Gaede pump it is, however, obtained quite white. The substance crystallised from water or dilute alcohol has the composition $C_{10}H_8N_4$, H_2O. The water of crystallisation is given off in a vacuum over phosphorus pentoxide at 100°, but under atmospheric pressure only at a considerably higher temperature. The anhydrous substance is very hygroscopic, and the hydrated substance was therefore analysed, after drying in a steam-bath until constant.

0·1408 g.; 0·0635 g. H_2O and 0·03093 g. CO_2,
0·00965 g. (Micro-Kjeldahl) gave 13·4 cc. N/70 NH_3,
0·0592 heated to 150° in air-bath until constant lost 0·0053 g.
0·1041 heated over P_2O_5 at 10 mm. and 100° lost 0·0090 g.

The molecular weight was determined by the micro-method due to one of us [Barger, 1904].

0·0508 g. in 0·81 g. ethyl alcohol was equivalent to 0·325 g. β-naphthol, whence M.W. = 193.

	C	H	N	H_2O	M.W.
Found	59·9	5·0	27·8	8·7, 8·9	193
Calc. for $C_{10}H_8N_4$, H_2O	59·4	5·0	27·7	8·9	202

Heated to 150° the hydrated substance sinters without melting; plunged suddenly into a bath at 160° it melts, but gradually solidifies again to the anhydrous substance, which melts at 235–238°. In a Gaede pump vacuum it sublimes with metal bath at 260°. It has not been possible to crystallise the anhydrous substance from non-aqueous solvents although the original crystalline form is preserved when the substance is dehydrated at 100° in a vacuum; exposed to the air, the anhydrous substance rapidly takes up rather more than a molecule of water.

The substance $C_{10}H_8N_4$ is a base, readily soluble in hydrochloric or acetic acid. The *nitrate* is, however, very slightly soluble and crystallises well from water; the *picrate* is hardly soluble in boiling water. The base has practically no effect on the blood pressure or at most, exerts a very slight pressor action.

Action of other reagents on α-hydroxy-β-glyoxaline-propionic acid.

By heating the above acid with baryta to 205°, Fränkel [1903] obtained a substance, very slightly soluble in water and melting at 216°, in quantity insufficient for analysis. We repeated this experiment on a much smaller scale but could not isolate Fränkel's substance nor the base $C_{10}H_8N_4$ and therefore cannot draw any conclusion as to their possible identity.

With syrupy phosphoric acid at 200° no reaction occurs. At 220–240° about one molecule of carbon monoxide is given off and the solution becomes intensely fluorescent; after removal of phosphoric acid by baryta and evaporation, the residue was found to consist, for the smaller part, of the fluorescent substance, slightly soluble in cold water, readily in alcohol, and for the larger part of a substance readily soluble in water but insoluble in alcohol. The second substance on heating with 85 % sulphuric acid to 180° is transformed into the crystalline base $C_{10}H_8N_4$.

Concentrated hydrochloric acid at 235° acts in much the same way as phosphoric acid, yielding a fluorescent mixture of substances.

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XXXII. FEEDING EXPERIMENTS WITH A DIETARY IN WHICH TYROSINE IS REDUCED TO A MINIMUM.

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(*Received July 25th, 1916.*)

Knoop's discovery [1910] that the α-amino-acids can be synthesised in vivo from non-nitrogenous organic compounds and ammonia is one of the most significant contributions to the physiology of metabolism. He and Kertess [1911] isolated the *d*-acetyl derivative of γ-phenyl-α-aminobutyric acid from the urine of a dog to which the sodium salt of the corresponding α-ketonic acid had been given subcutaneously, demonstrating the fact that the synthesis of the amino-acids from ketonic acids can be effected in the animal body.

As a result of the administration of γ-phenyl-α-hydroxybutyric acid to a dog, there was a rise in the excretion of the corresponding amino-acid, showing that the α-hydroxy-acids can be converted into the α-amino-acids.

Applying the method of liver perfusion to the problem, Emden and Schmitz [1910, 1912] have demonstrated that an analogous process may take place in connection with the synthesis of the normal protein constituents, such as tyrosine, phenylalanine and alanine from the corresponding ketonic acids, thus showing that the phenomenon of amino-acid synthesis is a general reaction.

The well-known observations of Loewi [1902] followed by the elaborate experiments of Abderhalden, Henriques and others, have conclusively proved that an animal can maintain itself and exhibit growth when free amino-acids replace the protein of the food.

It was the original intention of the present research to decide whether tyrosine can be replaced by its corresponding ketonic acid in a dietary in which the mixture of the hydrolysis products of caseinogen were the sole

source of nitrogen supply; it being assumed from the work of Abderhalden [1913] that, with a deficiency of this aromatic amino-acid, an animal is unable to exhibit normal nutrition. My preliminary experiments led, however, to the unexpected result that in the case of rats most of the individuals fed upon a dietary which contained at most a minimal supply of tyrosine, not only maintained their body weight, but also exhibited growth. The original intention of my research could not therefore be carried out, but the experiments which show the apparently unessential nature of tyrosine seem to be of interest, and are discussed in this paper.

On the quantitative isolation of Tyrosine from Caseinogen.

Before attempting the feeding experiments, an endeavour was made to isolate tyrosine quantitatively from the hydrolysis products of protein. By applying the method of recrystallisation to the amino-acid mixture from caseinogen hydrolysed by acid, in spite of adopting the details of the method of Abderhalden and Fuchs [1913], it was found to be extremely difficult to get rid of all substance yielding the Millon's reaction from the last filtrate, though no more crops of the characteristic crystals of tyrosine could be obtained therefrom by further crystallisation. In three experiments, in two of which hydrolysis was effected with sulphuric acid and in one with hydrochloric acid, the amounts of tyrosine obtained were respectively 3·45, 3·70 and 3·23 %. In comparison with the figure 4·5 %, which is provisionally accepted as the probably correct proportion of tyrosine in caseinogen, the above figures are evidently low, but, on the other hand, they are closely similar to those obtained by Osborne and Guest [1911], viz. 3·9, 3·2 and 3·4 %. These latter authors, in spite of careful work, were also unable to isolate the whole of the tyrosine present in caseinogen by direct crystallisation. It is evident that the solubility of tyrosine is profoundly affected by other amino-acids present in the mixture. In the hope of eliminating this influence, the following method was used. Caseinogen was boiled with sulphuric acid of 25 % strength for 72 hours, so that complete hydrolysis might be effected. After the removal of sulphuric acid, the solution was submitted to repeated crystallisation, the process of evaporation being always performed in vacuo. Even when the tyrosine had been separated as completely as possible, the filtrates still gave a Millon's reaction with apparently undiminished intensity. Starting with 30 g. of caseinogen, 1·02 g. of tyrosine were obtained in characteristic needles. To the filtrate an aqueous solution of mercuric

chloride was added; the first portions of the precipitate thus formed contained histidine [cf. Kossel, 1896], which was identified by means of its picrolonate. On the further addition of the precipitant to the now distinctly acid solution, more precipitate was obtained, which partly consisted of a sulphur-containing compound, probably cystine [cf. Suter, 1895]. These precipitates were filtered off, and the filtrate was so far saturated with mercuric chloride that the solution contained sufficient to give a yellow precipitate with baryta water; then, finely powdered baryta was added with continuous stirring until tyrosine was completely precipitated, as shown by the absence from the filtrate of Millon's reaction and of the modified diazo test for tyrosine [Totani, 1915]. Excess of baryta should be carefully avoided, as the tyrosine compound is apt to be redissolved in excess. In several experiments it was found that 50 g. of baryta were sufficient to precipitate all the tyrosine present in a litre of the solution. The precipitates were filtered off by suction and washed with baryta water; they were then suspended in water containing a slight excess of sulphuric acid, and decomposed with hydrogen sulphide. The filtrate and washings from the mercury sulphide and barium sulphate were concentrated; chlorine removed by means of silver sulphate, and the excess of silver by hydrogen sulphide. The whole solution was now made up to 500 cc. and contained approximately 5 % sulphuric acid. It was then precipitated with 10 % phosphotungstic acid solution. After leaving overnight, the precipitate was filtered off, and washed with 5 % sulphuric acid, until the washings no longer yielded Millon's reaction. The filtrate and washings were freed from an excess of phosphotungstic acid by baryta and subsequently from the latter by sulphuric acid. They were then submitted to repeated crystallisation. In this manner a further quantity of 0·23 g. of pure tyrosine was obtained. The amino-nitrogen content of this sample was estimated by means of van Slyke's method:

0·0554 g. gave 7·8 cc. N at 19° and 758 mm. = 8·03 % N.

Calculated for $C_9H_{11}O_3N$	Found
N 7·73 %	8·03 %

The tyrosine was therefore probably pure.

Since the filtrate from the last crystallisation still gave an intense Millon's reaction, an endeavour was made to crystallise more tyrosine, but without success. The total quantity of tyrosine isolated was 4·16 % of the protein used; considerably more than that obtained by direct crystallisation.

For the purpose of isolating tyrosine, therefore, it is advantageous to

remove other constituents as completely as possible from the hydrolysis mixture. Based on the observation that the more soluble fractions of a hydrolysis mixture will retain tenaciously material yielding a Millon's reaction, Gortner [1911] drew attention to the possible presence of some other aromatic phenolic body in the molecule of keratin. Guggenheim [1913] isolated dihydroxyphenylalanine from *Vicia fava*. This reacts with Millon's reagent, and it is possible that such a compound may exist in the protein molecule.

The above method of isolating tyrosine was beyond doubt more nearly quantitative than direct crystallisation, but it proved to be extremely tedious and unsuitable for preparing material for feeding experiments.

The results of numerous experiments agree in showing that tyrosine is liberated almost completely at a very early stage of tryptic digestion. Abderhalden and Voegtlin [1907] isolated 4·5 % from the tryptic products of caseinogen.

Advantage was taken of the above fact in a further endeavour to separate tyrosine with a greater completeness. Caseinogen was first digested with trypsin, and the liberated tyrosine filtered off. The filtrate was then submitted to acid hydrolysis in the hope of obtaining a further yield.

The tyrosine which separated from a thorough pancreatic digest of 60 g. of caseinogen was filtered off and purified by recrystallisation from water. The filtrate was concentrated on the water-bath and kept cool in a freezing mixture for several hours, and a further quantity of pure tyrosine obtained. Submitting the main solution to further crystallisation, it was found that a little more tyrosine could be isolated, but only after a non-Millon-yielding fraction, probably leucine, was first removed. The amount of tyrosine thus obtained was 2·27 g.

The whole solution was now made up to 400 cc., with the addition of sulphuric acid to the extent of 25 %, and boiled under a reflux condenser for 72 hours. After removal of the sulphuric acid, the solution was submitted to crystallisation until no more tyrosine could be isolated. The quantity of tyrosine isolated as the result of the secondary acid hydrolysis was 0·174 g.; the whole yield obtained amounting therefore to 4·07 % of the protein. In another analysis carried out with 100 g. of caseinogen, 4·02 g. of tyrosine were obtained.

In connection with the above analysis, the following bromination experiments were carried out. Millar [1903] first worked out a method for estimating tyrosine by measuring the quantity of bromine necessary to form the dibromo-substitution compound. Plimmer and Eaves [1913] modified the

method so that the estimation of even small amounts of tyrosine could be effected with accuracy; they applied the method in an endeavour to determine the tyrosine content of protein. It was found, however, that the presence of histidine and tryptophane, both capable of absorbing bromine, was a disturbing factor. The former could be removed by phosphotungstic acid, but, with regard to tryptophane, it was found that although this amino-acid was destroyed by boiling with acid, its decomposition products still reacted with bromine; accordingly, tyrosine could not be estimated by the bromine method in a solution containing the whole of the products of acid hydrolysis. Plimmer and Eaves therefore utilised a tryptic digest of the protein in which the tyrosine had been liberated at an early stage of the hydrolysis, while tryptophane was assumed to be still in combination.

It is clear that the bromination method cannot be applied for the purpose of determining how much tyrosine remains in solution after its isolation by direct crystallisation has been attempted. The amount of tyrosine separated may be measured, however, by determining the bromine absorbed by the whole hydrolysis mixture, and that absorbed after the tyrosine has been crystallised out; the difference should correspond to the amount of tyrosine obtained. This method I have applied. In a preliminary experiment the quantity of bromine absorbed by pure tyrosine was determined; this gave 1·803 g. of bromine for 1 g. of tyrosine. The figure is rather higher than the calculated value, 1·765 g., but agrees with the results obtained by Millar and by Plimmer.

The solution of hydrolysis products from 60 g. of caseinogen freed as completely as possible from tyrosine in the former experiment (solution I) was submitted to bromination. As control material the same quantity of caseinogen was treated in a precisely similar manner, but the tyrosine not separated (solution II). Each solution was made up to 500 cc. with the addition of a small quantity of hydrochloric acid to complete the solution of the amino-acids. 25 cc. of each solution were brominated.

	Bromine absorbed (in g.) by	
	Solution I	Solution II
	0·4200	0·6349
	0·4176	0·6334
Mean	0·4188	0·6341

Difference = 0·2153 for 3 g. protein,

∴ 7·176 for 100 g. protein.

Since 1 g. of tyrosine absorbs 1·803 g. of bromine, 7·176 g. correspond to 3·98 g. of tyrosine. From the solution I, tyrosine was, as previously stated, isolated to the extent of 4·07 % by the crystallisation method; the figures obtained agreed, therefore, within the limits of experimental error. It is clear, therefore, that tyrosine was removed from the amino-acid mixture to the extent of 4 % of the protein used. No attempt to estimate with accuracy the amount of tyrosine left in solution was successful.

Feeding Experiments with Caseinogen.

Method. All the experiments described in what follows were carried out on the lines adopted by Hopkins [1912] in his investigations dealing with the accessory growth factors. Young albino rats were employed; they were taken from a stock which had been fed upon oats and bread and milk. The origin and previous history of the animals were known. The experiments were carried out on strictly comparative lines. The experimental and control animals were so chosen that the groups compared were uniform in origin, sex and weight. They were fed and treated in an exactly similar manner, except with regard to the essential factor with which the investigation was concerned. Rats of the same sex were kept two together, bucks with bucks and does with does, in wire cages which were placed in a cellar of uniform temperature. The food was given in excess of consumption, and water was administered freely. Having regard to the results obtained by Hopkins and others, it is clear that when synthetic diets are fed, it is necessary to supply the accessory growth factors. In my experiments these were administered in the form of an alcoholic extract of milk, in a quantity such that the two rats received an amount corresponding with 1·5 cc. of fresh milk per diem. Observations were made on the change of weight and on the general well-being of the animals. In one experiment the survival period was determined. Nitrogen equilibrium was not determined. Henriques [1909] observed that the body weight of a rat might be maintained for some days, even when nitrogenous equilibrium no longer existed. Experiments carried out in this laboratory show, however, that such a relation is only temporary. The rats were weighed daily at a specified time, and the amount of food consumed during the previous 24 hours determined.

Experiment I.

In this the sole nitrogenous supply to the animal was an amino-acid mixture from which the tyrosine had been removed as completely as possible on the lines discussed in previous sections.

I have shown above that tyrosine can be more completely separated by making use of a combined tryptic and acid hydrolysis, followed by repeated crystallisation, than by the usual method. From 1 kilogram of the commercial caseinogen preparation known as "protene" 38·52 g. of tryosine were obtained in an apparently pure state. This figure is lower, of course, than that obtained as above from pure caseinogen, but making allowances for the impurities in "protene," the separation is, as a matter of fact, equally complete. The amino-acid mixture from which the tyrosine had been thus removed was used in preparing the diet for the experiment now to be described. The solution was evaporated nearly to dryness, and then a known quantity of potato starch was added. The whole mass was so far dried that the material could be easily powdered by means of a coffee mill. Fat and cane sugar were subsequently mixed in, and tryptophane to the extent of about 1·5 % of the protein which yielded the amino-acid mixture.

In the control diet, tyrosine was added to the extent of 3 % of the original protein.

As regards nitrogen content, the food was prepared in two forms: one containing 3 and the other 2 %. The actual composition of the diets was as follows:

	Food of 3 % N content	Food of 2 % N content
Products from hydrolysed protein and starch mixed (N 7·0 %)	43·0 %	28·6 %
Tryptophane	0·5	0·4
Starch	20·1	34·6
Cane sugar	21·2	21·2
Fat	12·5	12·5
Salts (ashes of oats and dog biscuit in equal proportion) ...	2·7	2·7

Before feeding, a small quantity of water was added, and rubbed in by hand, so that the food mixture was obtained in the form of lumps of adequate size and consistence for consumption by the rats. The dried alcoholic extract of milk was administered in a small daily ration as mentioned above.

Eight healthy male animals were employed: of these four were fed upon the basal diet only (set A), the other four received the tyrosine addition (set B). During the first 24 days all received the food containing 3 % of nitrogen; later, that containing 2 % was given with the intention of diminishing the tyrosine supply.

The result of the experiment may be summarised in the following figures:

Set	Rat No.	Duration of feeding on 3 % N food in days	Gain in weight in %	Duration of successive feeding on 2 % N food in days		Further gain in weight in %	
				First	Following	First	Following
A	1	24	38·6	10	7	0	7·5
	2	24	36·6	10	7	5·0	5·5
	3	24	34·6	10	—	9·5	—
	4	24	22·1	10	—	9·7	—
			Mean 32·9			6·0	
B	5	24	26·0	10	—	9·9	—
	6	24	30·9	10	—	7·4	—
	7	24	37·0	10	—	4·2	—
	8	24	26·1	10	—	8·5	—
			Mean 30·1			7·5	

The behaviour of individual animals was closely similar in all cases, and no marked difference between the two sets could be established. All the animals consumed the food equally well, and remained in good condition. Wet filter paper was given from time to time as a precaution, but no intestinal trouble was any time observed. From this experiment it may be concluded that rats may exhibit almost normal growth when the supply of tyrosine is diminished to an extremely small amount. This result, however, differs from that obtained by Abderhalden. In one of his experiments [Abderhalden, 1913] a dog was used; a preparation of the digestive products of caseinogen freed from tyrosine as completely as possible, by direct crystallisation alone, was given to the animal, which lost body weight to the extent of 750 g. in nine days. There was a further loss in the four following days, though this was partly caused by an insufficient intake of food. The loss of weight, however, was regained almost entirely when tyrosine was added to the previous dietary. Abderhalden therefore came to the conclusion that tyrosine is an essential factor in nutrition. As already mentioned, the original intention of the present research was to discover if tyrosine could be replaced in nutrition by the corresponding ketonic acid. When the work began there was, to the best of my belief, no suggestion in the literature for the plan of testing the possible nature of intermediary metabolic products by replacement of the original amino-acid in a diet. Since then, however, in the third edition of his Lehrbuch, Abderhalden [1915, 1] has made brief mention of an experiment carried out on rats by himself for the purpose of investigating the replaceability of tyrosine by its ketonic acid. In this experiment the quantitative separation of tyrosine was unsuccessful; the rat, however, was unable to

maintain its nitrogenous equilibrium on the reduced supply of tyrosine. The description of this research, mentioned incidentally in the text-book, is so brief that the details are not clear. It is obvious, at any rate, that Abderhalden's results were entirely different from mine[1].

It is, of course, an objection to my experiments that the actual amount of tyrosine left in the diet is uncertain. Abderhalden claims that in his amino-acid mixture not more than 0·2 % of tyrosine was left behind. It will be understood from what has gone before that to give an actual figure for this is impossible. It is extremely improbable, however, having regard to the methods that I used, that there was more tyrosine in the dietary used by me than in that employed by Abderhalden.

In the endeavour to avoid this difficulty, further experiments were carried out on a gelatin diet. In connection with the effect of tyrosine deficiency, it is important to remember the clear evidence obtained by Emden and Baldes [1913] that phenylalanine can be converted into tyrosine in the body. This was shown conclusively during the perfusion of the surviving liver with the former substance. An explanation, therefore, for the normal growth of animals with tyrosine deficiency may be sought in this conversion of phenyl-alanine, which is present in a considerable amount in protein, into the missing phenolic amino-acid. Such a rôle of phenylalanine in replacing tyrosine was suggested in a research of Abderhalden's [1914].

Feeding Experiments with Gelatin.

It has become so clear from recent researches that the efficiency of a dietary is not determined by the amount, but rather by the quality, of its nitrogenous constituents, that earlier researches upon the protein-sparing power of gelatin need not be referred to. So far back as 1876 Escher observed an improvement in the nutritive value of gelatin by the addition of tyrosine alone; but now that it is known that tryptophane, which is entirely absent from gelatin, is an essential factor for maintenance, any investigation made without the addition of this need not be considered. Kaufmann's [1905] paper is the first that needs attention. He found that protein could be replaced by gelatin, when tyrosine and tryptophane were added, to the extent of from one-third to one-half of the total nitrogen of the diet. Under these circumstances, the nitrogen balance was maintained. This experiment

[1] Abderhalden's full publication (1915, 2) was not seen until after this paper was written.

was carried out on a dog. In a further experiment performed upon himself, he reached the striking result that there was nitrogenous equilibrium when gelatin, with the addition of the absent amino-acids, replaced the whole of the protein of the diet. Kaufmann's experiment was repeated by Rona and Müller [1906] upon dogs. They could replace the protein by gelatin plus tryptophane and tyrosine, up to the extent of two-fifths of the total nitrogen, but not beyond this. They concluded, therefore, that the protein-sparing power of gelatin could not be enhanced by the addition of the missing aromatic amino-acid. Later on the problem was again attacked by Abderhalden and Manolin [1910], whose experiments confirmed Kaufmann's result. Because of the possible indigestibility of normal gelatin, these last observers employed predigested gelatin, to which several amino-acids, comprising not only those actually missing, but also those which are present in relatively small amount in the gelatin molecule, were added. In the dog, they found that this mixture could replace from three-fifths to two-thirds of the protein nitrogen in the food. Moreover, in a still later experiment, Abderhalden [1912] claims to have maintained a dog practically in nitrogenous equilibrium even on complete replacement of protein by the above mixture of gelatin and amino-acids. As a result of this experiment, Abderhalden assumes that a supply of the aromatic amino-acids is essential to life. As a matter of fact, however, until Abderhalden made the further reference in the text-book as mentioned above, to his study of tyrosine deficiency, there was really no evidence for the indispensability of an aromatic grouping, except in the case of tryptophane.

If a diet free from tyrosine is to be prepared from gelatin, it is essential that the gelatin should be pure. Among the many samples tested by me, the "gold label" gelatin of the French firm Coignet Père & Fils & Cie, with the mark "extra" was found to be practically free from other protein. Millon's reaction, however, was fairly distinct, when the gelatin was dissolved in water, with the addition of a little sulphuric acid, and excess of the reagent carefully avoided. Plimmer and Eaves [1913] submitted a gelatin[1] digest, previously freed from histidine, to bromination, and found that a certain amount of bromine was absorbed. They considered this due to the presence of tyrosine, and calculated its amount as 0·24 %. Recently, however, Siegfried and Reppin [1915] observed a marked difference in the amounts of halogen absorbed by gelatin according to the conditions under

[1] Plimmer and Eaves used also "gold label" gelatin as material, but did not mention the variety.

which bromination was performed. Intact gelatin absorbed much more than hydrolysed. They held that in the gelatin molecule some complex capable of combining with bromine might be present, which disappeared during acid hydrolysis. If this evidence be taken into consideration, the bromine value of gelatin, as found by Plimmer and Eaves, ought not to be regarded as due exclusively to tyrosine. Mörner [1899] states that even the purest gelatin gives a marked colour with Millon's reagent. Murlin [1907] endeavoured to purify gelatin by Kirchmann's method, but Millon's reaction remained undiminished in his final product. It is clear that at present we have no knowledge as to the nature of the Millon-yielding substance in gelatin. I had to be content, therefore, to use gelatin which, while certainly free from other protein, still yielded at least a slight colour with Millon's reagent. I shall now describe two feeding experiments, in one of which intact gelatin was used, and in the other the hydrolysed product.

Experiment (II) with non-hydrolysed gelatin.

A basal dietary was prepared in the following way. A jelly composed of 250 g. of pure gelatin and 420 g. of starch was dried to such a consistence that it could be passed through a mincing machine. This treatment was repeated until the whole could be sifted through a sieve of 1·5 mm. mesh, and the product was then completely dried. The other constituents, fat, sugar and salts, were finally mixed in the same proportion as in the diet of the previous experiment. The total nitrogen content of the basal dietary so prepared was 3·4 %. The calculated energy value was just over 5 calories per gram. The protein-free milk extract was given in a small ration as before. Three sets of animals were compared: the first set (C), were fed on the basal dietary alone; the second (D) received in addition tryptophane to the extent of 1·5 % of the gelatin used; the third set (E) were given, in addition to the tryptophane, tyrosine to the extent of 3 % of the gelatin used. The food was consumed equally well by all the rats, until a few days before their death. In all the sets, a steady loss of weight occurred from the beginning of the special feeding. The experiment was continued till all the animals had succumbed.

If set C be compared with set D, it will be seen that there was no difference either in the rate of loss of weight, or in the survival period. Even in the case of the rats fed upon the gelatin plus tryptophane and tyrosine, the

difference was but slight. An extension of the survival period by one week, is, I think, too short to be of much significance[1].

Set C N in food supplied by gelatin			Set D N in food supplied by gelatin + tryptophane			Set E N in food supplied by gelatin + tryptophane + tyrosine		
Rat No.	Survival period in days	Loss in weight in %	Rat No.	Survival period in days	Loss in weight in %	Rat No.	Survival period in days	Loss in weight in %
9	17	45·0	15	16	45·7	21	37	44·8
10	30	44·8	16	23	46·1	22	37	47·2
11	32	46·0	17	38	58·1	23	36	42·9
12	30	45·0	18	39	51·8	24	44	47·8
13	30	40·8	19	27	45·5	25	33	44·2
14	28	48·0	20	24	44·8	26	32	47·3
Mean	27·8	44		27·8	48·6		34·8	45·7

Average loss of weight 46·4 %.

As the following experiment shows, the failure to obtain any improvement by the addition of the missing amino-acids was not due to the lack of their significance in metabolism, but to the fact that the intact gelatin itself is very badly absorbed.

Experiment (III) with hydrolysed gelatin.

In this experiment, the products of the acid hydrolysis of gelatin, were employed as the source of nitrogen supply in the basal dietary. The food was prepared exactly as in the case of the caseinogen preparation of Experiment I. The total nitrogen content was brought to the same value as that of the non-hydrolysed gelatin diet of Experiment II.

Six sets of animals were compared; in each set four rats (two bucks and two does) were employed. The rats of the first set (F) were for comparison fed upon the non-hydrolysed gelatin diet, as used in Experiment II. All the other sets were put upon the hydrolysed gelatin food. The second set (G) had this basal diet alone; the third (H) the same with the addition of tryptophane; the fourth (I) with tryptophane and tyrosine. To the last two sets (J) and (K) histidine and cystine were given in addition to the above, in the hope of still further enhancing the nutritive power of the gelatin. Cystine, of course, is missing from gelatin, and histidine occurs in a very small amount only. In set (K) tyrosine was replaced by synthetic phenylalanine.

[1] It may be mentioned that three of the rats from set E displayed peculiar symptoms some 12–24 hours before death. Paralysis began in the hind-legs, and extended rapidly over the trunk to the fore-legs, so that the animal could no longer move. Clonic and tonic spasms then followed and death occurred in a few hours. A post-mortem examination revealed nothing remarkable. As to the cause of these symptoms, I have no suggestion to offer, but it does not seem probable that they were accidental. They certainly did not suggest a tetanus infection.

The histidine was obtained from ox blood by the method of Knoop [1907], the hydrochloride obtained being neutralised in solution with sodium carbonate before mixing with the food. The cystine was obtained from hair; the phenylalanine was prepared by the method of E. Fischer, the product used melting at 263°. The amounts of tryptophane and tyrosine added were the same as in the previous experiment. Histidine and cystine were added to the extent of 2 and 1 % of the gelatin respectively. As regards phenylalanine, though it is known that the *d.l.* compound can be completely consumed in the organism of the dog [Schotten, 1883; Knoop, 1905], and also that it increases the homogentisic acid formation, as much as the active compound [Abderhalden, Bloch and Rona, 1907], yet having regard to the general evidence for the necessity of supplying the natural form of amino-acids, the racemic phenylalanine was employed in quantity double that of the tyrosine it was to replace.

Set F				Set G			
Rat No. and sex	Change in weight in %	Duration of feeding in days	Remarks	Rat No. and sex	Change in weight in %	Duration of feeding in days	Remarks
27 ♂	−34·4	31	alive	31 ♂	−18·2	31	alive
28 ♂	−44·4	26	dead	32 ♂	−19·6	31	,,
29 ♀	−33·8	31	alive	33 ♀	−17·9	31	,,
30 ♀	−35·3	31	,,	34 ♀	−17·5	31	,,
Mean	−36·9				−18·4		
Set H				Set I			
35 ♂	+ 8·5	31	alive	39 ♂	−17·1	31	alive
36 ♂	−15·0	31	,,	40 ♂	− 8·4	31	,,
37 ♀	+ 0·4	31	,,	41 ♀	+ 6·4	31	,,
38 ♀	− 8·4	31	,,	42 ♀	+ 1·4	31	,,
Set J				Set K			
43 ♂	−26·2	22	alive	47 ♂	+11·3	22	alive
44 ♂	+ 5·2	22	,,	48 ♂	+ 5·8	22	,,
45 ♀	+ 3·2	31	,,	49 ♀	−14·6	31	,,
46 ♀	− 0·9	31	,,	50 ♀	− 0·9	31	,,

If now set (F) be compared with set (G), it becomes very clear that the previously hydrolysed gelatin can be much more readily employed by the rat than the non-hydrolysed, since the loss of weight for the given period is reduced to a half. The difference does not depend upon the quantity of food consumed. The following figures which give the average consumption per 100 g. body weight show that closely similar amounts of food were eaten by the animals in both sets.

A somewhat diminished consumption in the later periods in the case of the set taking the non-hydrolysed gelatin, may be considered as the effect

and not the cause of the loss of weight, which was much more rapid than in the case of the animals taking the hydrolysed gelatin [cf. Hopkins, 1912]. It seems clear that the intact gelatin is badly digested and absorbed by the rat, and as already claimed, this fact explains the failure to obtain good results upon the addition of the missing amino-acids in the previous experiment. The results in the case of set (H) were remarkable. The treatment of the animals was exactly similar to that of set (G), save that a little tryptophane was given. Of four rats, two were not only able to maintain their weight, but also exhibited some growth. The general condition of these animals also remained satisfactory. The condition of the other two rats of this set was also for a long time much better than that of rats receiving no tryptophane. These two animals, it is true, lost weight; but in such experiments a positive result is clearly of the greater significance. It may be concluded, therefore, that rats can at any rate maintain themselves upon the hydrolysed products of gelatin when tryptophane alone is added.

	Set F			Set G		
	Food* eaten by			Food* eaten by		
Duration of feeding	Rats 27 and 28	Rats 29 and 30	Mean of loss of weight	Rats 31 and 32	Rats 33 and 34	Mean of loss of weight
1–3 day	6·4 g.	7·9 g.		5·7 g.	5·5 g.	
4–6 „	7·4	7·1	12·4 %	8·4	8·8	8·3 %
7–9 „	8·3	7·6		7·9	8·9	
10–12 „	7·1	7·1		7·4	7·6	
	Mean 7·3 g.			7·5 g.		
13–15 day	7·1 g.	5·8 g.		8·4 g.	7·3 g.	
16–18 „	6·7	5·8	22·2 %	7·7	7·3	4·9 %
19–21 „	6·4	5·8		7·9	7·1	
22–24 „	7·2	6·2		7·8	6·4	
	Mean 6·4 g.			7·4 g.		

* The average daily food consumption per 100 g. weight during the experimental period.

In set (I) tyrosine was given in addition to tryptophane, with scarcely any effect. Sets (H) and (I) closely agree. If tyrosine improves the nutritive value of gelatin, its effect is certainly much less than that of tryptophane.

In the last two sets (J) and (K), which received histidine and cystine in addition to tryptophane and tyrosine (or phenylalanine), the condition of the rats was somewhat more satisfactory than in the other sets. I think this experiment may be taken as showing that the complete replacement of protein by hydrolysed gelatin plus the missing amino-acids is quite possible. If tyrosine be necessary for maintenance and growth, phenylalanine seems able to act as raw material for its formation.

Discussion of Results.

Perhaps the most important point brought out in the above experiments is the fact that the rat can maintain itself and even gain weight on a hydrolysed gelatin diet, with the addition of the single amino-acid, tryptophane. Based upon the feeding experiments carried out with Miss Willcock, Hopkins [Willcock and Hopkins, 1906] has suggested that the protein constituents may be utilised not only for tissue formation, or structural maintenance, but in a more specific and direct manner, as, for instance, in the elaboration of essential substances such as adrenaline or other hormones. We have no direct evidence at present to show that adrenaline is derived from tyrosine, though the close resemblance in the chemical constitution of these two substances makes it very tempting to believe that the former is the precursor of the latter.

When animals are observed to grow normally upon food containing most minute quantities of tyrosine (Experiment I, set (A)), it is almost certainly because phenylalanine is capable of replacing tyrosine and serving as its precursor. The amount of phenylalanine in protein is sufficiently great to justify this belief. As regards the result of the experiment in which there was maintenance with tryptophane only added to the gelatin hydrolysis products, some further explanation seems necessary. In this case the total amount of the aromatic groups in the diet was very small. The minimal requirement of the animal in this respect is of course not yet known. It appears probable, however, that the amount of phenylalanine in the gelatin dietary is too small to cover the requirement, and one has to think of the possibility of the synthetic formation of the benzene ring in the body. Recent advances in the physiology of intermediary metabolism have suggested that the synthetic power of the body is much greater than was previously thought. With respect to the simpler aliphatic amino-acids, such as glycine and alanine, it may be taken as already proved that they can be built up in the animal body. As regards the synthetic formation of the more complex amino-acids, such as histidine and phenylalanine, proof is yet lacking. As is well known, Knoop and Windhaus [1905] have suggested that histidine may be formed in the organism from methyliminazole and glycine, by means of an oxidative synthesis, the former substance arising from glucose and ammonia. As regards the synthesis in the organism of amino-compounds derived from benzene or phenol there is, so far as I know, nothing yet known. Nevertheless, the above-mentioned fact that the animal can subsist without suffering

tissue breakdown on hydrolysed gelatin and tryptophane alone, points, I think, to the possibility of a synthesis of a benzene nucleus. On the other hand the indole ring, as many experiments in this laboratory seem to show, cannot be dispensed with from the diet.

Summary.

(1) Tyrosine could not be isolated quantitatively from the hydrolysis products of caseinogen.

(2) When the removal of tyrosine from the amino-acid mixture is made as complete as possible, and is effected to an extent which certainly leaves only minimal quantities of this constituent, there appears to be no effect upon the nutritive value of the amino-acid mixture.

(3) In the case of the rat, the nutritive efficiency of gelatin is greatly increased by previous hydrolysis. Gelatin when fed intact appears to be badly digested and absorbed.

(4) The possibility of completely replacing the protein of a diet by hydrolysed gelatin, plus certain amino-acids, is confirmed.

(5) Some evidence is offered that the addition of tryptophane alone to the hydrolysis products obtained from pure gelatin made these efficient in maintaining the nutrition of animals.

The subject of the present investigation was suggested to me by Professor F. G. Hopkins who is responsible for the feeding experiments and to whom I am greatly indebted for valuable advice.

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[1] Owing to the present circumstance, this book was only obtained at the end of October, 1915, when the present investigation was nearly finished.

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XXXIII. A GENERAL METHOD OF ESTIMATING THE RELATIVE TURBIDITY OR OPACITY OF FLUID SUSPENSIONS INCLUDING BACTERIAL EMULSIONS.

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(*Received July, 24th, 1916.*)

Methods of estimating the turbidity or opacity of fluids have been employed by chemists for some years past. T. W. Richards [1895] was the first to describe an apparatus, the "nephelometer," designed for this purpose. Later P. A. Kober [1913] found it possible to estimate turbidity with a modified Duboscq colorimeter; and the same instrument has been employed in varying modifications by Marriott [1913], Folin and Dennis [1914], W. R. Bloor [1914] and others. Dreyer and Hansen [1907] made use of the turbidity, or rather the "clarification," of fluids in an investigation of the effects of light upon enzymes etc. They carried out their experiments in dwarf test-tubes, arranged in series to contain diminishing concentrations of the active substance in a constant volume of substrate, and they found that, when dealing with a turbid substrate, the extent of the reaction could be ascertained by reading the degree of clarification of the fluid. This procedure, although used for a quite different purpose, embodies the main features of the method described in this paper.

Measurements of the turbidity or opacity of fluid suspensions have hitherto lain almost exclusively within the province of pure or applied chemistry, and no great effort has been made to employ accurate methods of this kind in the service of bacteriology. Nevertheless there are numerous bacteriological procedures which would be facilitated by an accurately quantitative method of turbidity estimation; and for one process in particular

it is absolutely essential to use a method of this kind. We refer to standardisation of agglutinable cultures. During the last twelve months the particular macroscopic method of agglutination, described by one of us [Dreyer, 1909] has come widely into use. The most essential feature of the method is the employment of sterile formalised and standardised bouillon cultures of the bacteria. The standardisation process is a double one, and concerns firstly the opacity or turbidity of the culture, and secondly its specific sensitiveness to agglutination.

The second part of the process does not concern us here [see Davison, 1916]. The measurement of the turbidity of the cultures is of great importance, because upon it depends the ease and accuracy with which their relative sensitiveness to agglutination can be estimated; for it is very difficult to compare a thin culture with a thick one, or *vice versâ*, whereas the sensitivity-estimation of cultures of equal thickness presents, as a rule, little difficulty. The turbidity-test does not necessarily provide an accurate measurement of the number of bacteria in a bouillon culture, for the total turbidity will include any initial opalescence of the bouillon in which the bacilli are grown. The difference in sensitiveness of the cultures is indicated by the sensitivity-constants of the individual cultures.

The first apparatus which we employed for turbidity-standardisation was the Duboscq colorimeter modified in a new way to serve our special purpose. Within certain limits this yielded very satisfactory results, but a serious obstacle to uniformity and certainty of the readings was encountered in the fact that our veal-bouillon cultures were not of uniform colour. The deeper the colour of the fluid, the lower were the readings for a given degree of turbidity. Various means of overcoming this difficulty suggested themselves, and of these one promised considerable success; viz. the addition of a strong deep-brown peptone solution to the paler of the two fluids to equalise the colours. But after a considerable number of these experiments had been carried out the method was discontinued and replaced by the much simpler and more accurate method described below.

General Description of Method. A graduated series of dilutions of each fluid is made in miniature test-tubes in practically the same manner as in the determination of the agglutinability. By artificial light against a dark background the turbidities of a number of tubes of one series are matched in succession with tubes of the other series. Each reading provides figures from which the relative turbidities can be calculated, and by taking the average of a number of readings an accurate ratio is obtained.

Details of Method. Apparatus. Almost any variety of dwarf test-tube or agglutination-tube may be used. It is essential to have at hand a fairly large supply of tubes, so that a selection may be made of tubes of practically equal internal diameter. The tubes must be quite clean and their surface must be free from scratches. We have been employing for the purpose two varieties of tubes which are made by Baird and Tatlock, Ltd. for the National Medical Research Committee, viz. (1) A thin-walled dwarf test-tube about 7·0 cm. long and 0·95 to 1·0 cm. in diameter. (2) A smaller "agglutination tube" 5·7–5·8 cm. in length, having an internal diameter of 5·5–6·2 mm. When the greatest possible accuracy is desired, it is best to use the larger form of tube and to measure the fluids with a 2·0 cc. pipette, accurately graduated in hundredths of a cc. But for ordinary purposes the small agglutination tubes may quite well be employed, and the dilutions made with a dropping pipette. In this latter method there is a certain error due to the difference in size of the drops of 0·85 % NaCl solution and of culture. About 17–18 drops of culture are equal to 15 drops of saline. But in dealing with small volumes of fluids, it is open to doubt whether measurements with a small graduated pipette are any more accurate than this.

Technique (when small agglutination-tubes and a dropping pipette are employed). Two sets of eleven gauged tubes are placed in a suitable stand. Into the tubes of each set the different fluids are measured as follows:

Tube	Saline	Culture
(1)	0	20
(2)	4	16
(3)	8	12
(4)	10	10
(5)	12	8
		Culture diluted 1 in 2
(6)	6	14
(7)	8	12
(8)	10	10
(9)	12	8
(10)	14	6
(11)	16	4

This series of dilutions is similar to that described for the serum-dilution in the standardisation of agglutinable cultures, only multiplied by two. It will be seen that the range of dilutions in the series is from 1/1 to 1/10, and that the progressively diminishing quantities of culture are always made up to the same volume with saline. The mixtures in the tubes are well shaken with the finger over the mouth of the tube, and the readings are then taken.

Readings. Artificial light and a black background are essential. It has

been our custom to use a frosted electric bulb with a large green shade, and some pieces of dull black boarding or paper placed on the table below the lamp and propped up behind it. A darkened room, though not absolutely necessary, adds considerably to the ease and accuracy of the readings. The lamp shade is inclined so that the bulb is just hidden from the eye, and the tubes to be compared are held up against the edge of the shade. Before reading, the outside of the tubes must be wiped with the moistened corner of a glass-cloth and then polished with the dry part of the cloth. From one series a tube is chosen at random which we call the "standard" tube for the first reading. With this the tubes of the other series are compared one after the other until one is found which matches it in turbidity. It will frequently be found that there is no tube in the second series which gives an exact match, but that there is one tube which is a little more turbid, while the next lower is a little less. In this case the standard tube is recorded as lying between those two tubes of the other series.

Moreover it is usually possible to judge whether it is nearer to one than to the other. If so, we make a mark or marks under the number of the tube to which it is nearer. Five or six readings should be made in this manner, taking a number of tubes in succession as standard. It is well to read in both directions; *i.e.* to take tubes from each series as standard. Now, since we know the dilution of the culture in every tube, it needs only a simple calculation to arrive at the relative turbidity of the two cultures. Records of typical routine estimations are given in Tables I to IV. Experiment III was performed with small tubes and a dropping pipette; I and II were made with larger tubes and a 2·0 cc. pipette. When the latter procedure is followed there is no need to make a 1 in 2 dilution of the culture for the latter part of the series. Moreover twelve tubes may be used with advantage instead of eleven.

Thus: culture (cc.) 1·0, 0·8, 0·6, 0·5, 0·4, 0·35, 0·3, 0·25, 0·2, 0·17, 0·13, 0·10. Water (cc.) 0·0, 0·2, 0·4, 0·5, 0·6, 0·65, 0·7, 0·75, 0·8, 0·83, 0·87, 0·9. The range of dilution is the same as before; and the only difference is that the series is fuller by one tube at the right-hand end, which renders readings in this neighbourhood more accurate.

Moderate variations in the colour-intensity of the fluids do not appreciably affect the accuracy of the readings, for the progressive dilution renders such differences negligible at an early point in the series. If one of the fluids under comparison is so thick that the range of dilutions described proves insufficient to allow a comparison with the other fluid; then a further series

of tubes must be set up in precisely the same manner, but using the thicker fluid diluted (1 in 10 or 1 in 20) instead of at full strength.

In practice it is often convenient to make a rough estimate of the relative thickness of the fluids before proceeding to the complete test. This is easily done by taking a small quantity of the two fluids in two tubes of equal calibre, and diluting the thicker fluid until the two show approximately equal turbidity. From this rough test it is easy to make an approximate calculation of the relative thickness of the fluids. If one fluid appears to be four or five times thicker than the other, it is best to dilute it suitably before putting up the test-series.

TABLE I.

Estimation of relative turbidities of fluids A and B.

A is a stock "standard" culture.

B is a fresh, undiluted, formalised bouillon culture of *B. Typhosus.*

Technique: graduated 2·0 cc. pipette and large tubes.

Readings		Actual quantities of culture in the matched tubes		Ratio $\frac{\text{quantity of } A}{\text{quantity of } B}$	Deviation from mean in %
Series *A* Tube	Series *B* Tube	*A*	*B*		
2 matches	7	0·80 cc.	=0·30 cc.	2·67	0·37
3–4	„ 9	0·53*	=0·20	2·65	1·12
4–5	„ 10	0·46	=0·17	2·71	1·12
2–3	„ 8	0·67	=0·25	2·68	0·00
6–7	„ 11	0·35	=0·13	2·69	0·37
				Mean 2·68	Mean error 0·60 %

* This figure is obtained by interpolation. The reading 3–4 signifies "between tubes 3 and 4, but rather nearer 4." Now tube 3 contains 0·60 cc. and tube 4 contains 0·50 cc.—so we may take the figure to be a little less than 0·55, viz. 0·53.

The average-figure given at the foot of the third column is an expression of the fact that 1·0 cc. of *B* gives the same turbidity-reading as 2·68 cc. of *A*, when both are diluted to the same volume. If we now wish to dilute *B* to the same turbidity as *A*, all we have to do is to add to each 1·0 cc. of *B* 1·68 cc. of diluting fluid.

The readings vary, as a rule, about 3 % to 5 % on either side of the mean, and it is uncommon to find a reading with an error so great as 10 %. Such a close correspondence of the individual readings, each of which is in itself an experiment, is sufficient evidence of the trustworthiness of the method.

TABLE II.

Estimation of relative turbidities of fluids A and B.

A is a stock standard culture.

B is a fresh, undiluted, formalised broth culture.

Technique: graduated 2·0 cc. pipette and large tubes.

Readings: Series *A* Tube	Readings: Series *B* Tube	Actual quantities of culture in the matched tubes: *A*	Actual quantities of culture in the matched tubes: *B*	Ratio $\frac{\text{quantity of } A}{\text{quantity of } B}$	Deviation from mean in %
5 matches	9	0·40 cc.	=0·20 cc.	2·00	0·50
2 „	5	0·80	=0·40	2·00	0·50
6 „	10	0·35	=0·17	2·06	2·49
3 „	7	0·60	=0·30	2·00	0·50
7 „	10–11	0·30	=0·15	2·00	0·50
				Mean 2·01	Mean error 0·90 %

Calculation for diluting *B* to the same turbidity as *A*:

1·0 cc. of *B* = 2·01 cc. of *A*.

Therefore, to each 1·0 cc. of *B* add 1·01 cc. of diluting fluid.

TABLE III.

Method: small Dreyer's agglutination tubes and dropping pipette.

A is a stock standard culture.

B is a fresh, undiluted formalised bouillon culture.

Readings: Series *A* Tube	Readings: Series *B* Tube	Actual quantities of culture in the matched tubes: *A*	Actual quantities of culture in the matched tubes: *B*	Ratio $\frac{\text{quantity of } A}{\text{quantity of } B}$	Deviation from mean in %
4 matches	9	0·500 cc.	=0·200 cc.	2·500	0·16
3 „	8–9	0·600	=0·245	2·450	2·16
2 „	6–7	0·800	=0·315	2·540	1·44
1 „	5–6	1·000	=0·395	2·530	1·04
6 „	10–11	0·350	=0·140	2·500	0·16
				Mean 2·504	Mean error 0·99 %

Although we have paid special attention to the use of this method in the preparation of standard agglutinable cultures, the method itself is not limited to any such special use. As an instance of its application in quantitative chemistry we give in Table IV an experiment in the estimation of minute quantities of silver chloride in suspension or colloidal solution.

The close correspondence between the values obtained by our turbidity-method and the values calculated from the known strengths of the solutions demonstrates that this simple technique is at least as accurate as the more elaborate methods of turbidity-estimation hitherto employed in the quantitative measurement of dilute inorganic solutions.

Description of Experiment shown in Table IV. The estimation of the relative strength of two dilute $AgNO_3$ solutions—by quantitative conversion into AgCl and turbidity-measurement. The $AgNO_3$ solutions were kindly prepared for us by Mr N. V. Sidgwick, and were labelled A = between N/100 and N/1000, and B = between N/1000 and N/10,000. Their true concentrations, which were unknown to us until after the experiment was completed, were:

$$A = 0{\cdot}001254 \text{ N } AgNO_3,$$
$$B = 0{\cdot}0004013 \text{ N } AgNO_3.$$

After a rough preliminary experiment, solution A was diluted one in four, to bring it down to a strength sufficiently near that of solution B. A thus diluted is referred to as A (0·25).

To 10 cc. of solution A (0·25) 10 cc. of 0·85 % NaCl solution was added.

To 10 cc. of solution B 10 cc. of 0·85 % NaCl solution was added.

The mixtures were kept for four hours in a cool and dark place, and then the resulting suspensions (or colloidal solutions) of AgCl were subjected to a turbidity-estimation in the usual manner. Both solutions showed a bluish opalescence without any visible trace of flocculation. Large tubes and a graduated 2·0 cc. pipette were employed for the measurements.

TABLE IV.

Turbidity-estimation of two silver chloride suspensions (or colloidal solutions).

A and B are two $AgNO_3$ solutions, converted quantitatively into AgCl (see text).

Readings A (0·25) B Tube	Actual quantities of AgCl suspensions A (0·25)	B	Ratio observed $\frac{0{\cdot}25A}{B}$	Deviation from mean in %	Ratio calculated $\frac{0{\cdot}25A}{B}$
1 = 2 – 3	100	76	1·316	+2·41	
3 = 4 – 5	60	47	1·277	−0·62	$A = 0{\cdot}001254$ N $AgNO_3$
5 = 6 – 7	40	33	1·212	−5·68	$B = 0{\cdot}0004013$ N $AgNO_3$
7 = 8	30	25	1·200	−6·61	$\frac{0{\cdot}25A}{B} = \frac{{\cdot}0003135}{{\cdot}0004013} = \frac{1}{1{\cdot}280}$
7 – 8 = 9	27	20	1·350	+5·06	
5 – 6 = 7	38	30	1·267	−1·40	
3 – 4 = 5	52	40	1·300	+1·17	
2 – 3 = 4	68	50	1·360	+5·84	
			Mean 1·285	Mean error 3·60	1·280

Percentage divergence of observed from calculated ratio −0·39.

Appendix.

Gauging of the Tubes. For the selection of tubes of almost equal bore, a gauge is necessary. The following type has been found very convenient (Fig. 1). From a thin strip of brass a piece about an inch long is cut in the shape of a longitudinal section of a long truncated cone. The narrower end has the width of the minimum internal diameter allowable for the selected tubes (see below) and the wider end, to which a brass rod is soldered as a handle, has the width of the maximum internal diameter permissible. The gauging of the tubes consists in choosing those tubes whose mouths permit the entry of the narrower end of the gauge but refuse to admit the broader end. (A temporary but perfectly serviceable gauge of this kind can quickly

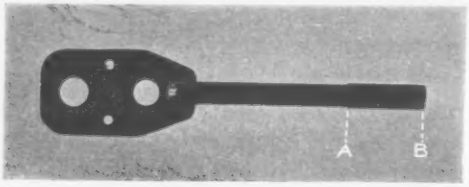

Fig. 1. Composite gauge for agglutination-tubes and dropping pipettes.

At one end of the instrument is seen the maximum (*A*) and minimum (*B*) gauge for the internal diameter of the agglutination-tubes. This is the only part that is described in the text. The holes in the plate at the other end are for the gauging of the external diameters of the tubes and of the capillary end of the dropping-pipettes. This part of the instrument is not essential for the method we have described.

and easily be cut from a small strip of thin wood to any designed measurement.) For the larger tubes the maximum and minimum measurements of the gauge may conveniently be made 9·3 mm. and 9·0 mm. respectively; and for the smaller tubes 5·7 mm. and 6·0 mm. respectively.

Although this type of gauge measures only the mouth of the tube, and gives no information about its lower end, we have not found that this introduces any appreciable error, since the tubes are not long enough to show any considerable variation in width from end to end.

It is hardly necessary to say that the more uniform the bore of the selected tubes is, the greater will be the accuracy of the turbidity-readings.

SUMMARY.

A description has been given of a simple and accurate method of measuring the relative turbidity or opacity of two fluids; and its special application to the standardisation of agglutinable cultures has been described in detail.

The method is of general application in all chemical and biological investigations which require the measurement of turbidity.

The technique is extremely simple and no special or expensive apparatus is needed.

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XXXIV. ANAPHYLAXIS TO THE SEPARATED PROTEINS OF HORSE-SERUM.

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(*Received July 31st, 1916.*)

The earliest attempt to examine the part played by the different serum proteins in anaphylaxis to a foreign serum was made by Gay and Adler [1908]. Gay and Southard had based a theory of the anaphylactic condition on an erroneous interpretation of the phenomenon of passive anaphylaxis. This theory explained the sensitive condition as due to a remnant of the foreign serum, introduced at the sensitising injection, and still present in the system. Clearly it might be expected that the remainder thus conferring sensitiveness would belong to a different fraction of the serum protein from that which, at the second injection, acted as a poison. In accordance with this expectation, Gay and Adler found that the euglobulin fraction of a serum had great power of producing sensitiveness to the serum from which it was separated, but no toxic action when reinjected into the sensitised animal. Taking further fractions they observed a progressive decline of sensitising power, and increase of toxicity to the sensitised animal, as the proportion of ammonium sulphate needed for precipitation became greater; so that the highest fraction, the albumin, had practically no power of producing sensitiveness, but a maximum toxicity for animals sensitised with the whole serum, or with the lower fractions. These conclusions are in complete disaccord with the later results, obtained by various investigators, on anaphylaxis to pure proteins, of animal or vegetable origin. Such results have indicated a considerable degree of specificity of the reaction, not only for the species from which the protein originated, but even for pure proteins separated from an organ or tissue.

Doerr and Russ [1909, 1], repeating Gay and Adler's experiments with

more attention to quantitative detail, obtained an entirely different result. Like Gay and Adler, Doerr and Russ worked with guinea-pigs, and separated the proteins of the ox or horse-serum used for sensitising by fractional precipitation with ammonium sulphate. They found, however, that sensitising power, and toxicity for the sensitised animal, declined in a parallel manner, with increase of the proportion of ammonium sulphate necessary for precipitation; so that the euglobulin fraction exhibited the maximum anaphylactic activity in either direction, the albumin being, in both respects, practically inert, and the pseudoglobulin occupying an intermediate position. Doerr and Russ's results are in conformity with the general tendency of the evidence obtained with other proteins. The only anomaly is the failure of the albumin fraction to act as an anaphylactic antigen. In a later paper [1909, 2], however, they showed a parallel failure of serum-albumin to produce a precipitin when injected into the rabbit, and, in conformity with their view of the identity of anaphylactic antibody with precipitin, a failure of serum from a rabbit so injected to confer passive anaphylaxis on the guinea-pig.

This question of the antigenic properties of the separate serum proteins, with regard to precipitin formation, has been examined by several other investigators, with results which show the widest possible variation, both in respect of the relative efficacy shown by the different proteins in exciting precipitin formation, and of the specificity to the individual proteins of the precipitins obtained. Some agree with Doerr and Russ as to the absence of a precipitin reaction to serum albumin; others, while obtaining a precipitin by injection of albumin, find that it precipitates even more strongly with globulin than with the albumin exciting its production. Doubtless a good deal of the variation is due to the varying degrees, in which the different methods employed effected a clean separation of the different proteins. The most convincing account is that given by Hunter [1905], whose paper may be consulted for the earlier literature. Hunter found that each of the serum proteins, euglobulin, pseudoglobulin, and albumin, was capable of exciting precipitin formation, and that the precipitin obtained in each case reacted most strongly with the protein used in its production, but in a modified degree with the others also; in other words, each precipitin showed a relative, or quantitative, but not an absolute, or qualitative specificity for its own protein.

Certain points in the behaviour of the serum-proteins as anaphylactic antigens seemed to deserve further investigation. Doerr and Russ's

description of the progressive weakening of the anaphylactic effect in passing from the euglobulin to the albumin fraction, the pseudoglobulin being intermediate in activity, suggests a possibility that euglobulin may be the only protein in serum capable of producing the reaction, and that the weaker reaction of pseudoglobulin may be due to incomplete separation. The point is not without practical significance, as well as theoretical interest. The antibody of immune horse-serum has been shown to be associated with the pseudoglobulin fraction, and the various methods of artificial concentration have aimed at eliminating the albumin and the euglobulin; but the details of the processes in actual use would seem to ensure a more thorough exclusion of the former. It is clear, on the other hand, if the euglobulin is the anaphylactically active constituent, and the albumin indifferent in this respect, that the aim of any process for purifying antitoxin should be as thorough a removal of euglobulin as is possible in large-scale working.

Methods.

We have confined our attention to horse-serum, specimens of the three proteins from which were available for use. Those which we used in the majority of the experiments were prepared by one of us for a chemical investigation, the results of which have already been published, together with the details of the methods of preparation [Hartley, 1914]. The euglobulin used was that prepared by Panum's method, and probably made a nearer approach to complete purity from the other proteins than did the specimens of pseudoglobulin and albumin, although these were probably as pure as repeated salting-out could make them. An additional specimen of the pseudoglobulin of horse-serum was kindly placed at our disposal by Dr H. Chick, who, after the usual separation by ammonium sulphate, had further purified it by continuous dialysing, with removal of the euglobulin thrown out of solution, during some months. Both the samples of pseudoglobulin were "euglobulin-free," in the sense that the ordinary methods of detection and separation would fail to discover euglobulin in them. But the quantity of a protein needed for sensitisation of the guinea-pig has been shown to be so minute, that it cannot be safely assumed that either was free from such traces of euglobulin as might be perceptibly effective in this direction. The same may be said with regard to a possible contamination of the albumin with minimal remnants of pseudoglobulin. In addition to the specimen above mentioned, a sample of albumin prepared in a different way was used

in several experiments. This was a crystalline albumin prepared by Hopkins and Pinkus's [1898] method, and recrystallised three times, only that fraction being taken of each crop which consisted wholly of large well-formed crystals. This was dialysed free from ammonium sulphate and dried to a scale in vacuo. A portion of it was, for another purpose, dissolved in water, precipitated with cold acetone and ether, extracted for three days with warm ether, and finally taken up in water and recrystallised by Hopkins' method, yielding particularly well-formed crystals. These were again dialysed, scaled, and kept dry. Neither of these preparations showed any notable difference in action from the albumin-fraction obtained by salting-out in the ordinary way.

Most of our experiments were made on guinea-pigs, which were sensitised by hypodermic injection of a small dose, either of whole fresh serum, or of a solution of one of the separated protein preparations. After an interval, varying from 12 up to 31 days, the sensitiveness of the uterine plain muscle to two or more of the separated proteins was tested by the method previously described by one of us [Dale, 1912]. The use of this method has the definite advantage, over experiments made on a series of similarly prepared animals, that no allowance need be made for individual differences of sensitisation. The reaction of one horn of the uterus is tested with one of the pure proteins, and subsequently the reaction of the other horn, which can be regarded with some certainty as equivalent in specific sensitiveness and physiological condition to the first, is tested similarly with another of the pure proteins. Moreover the degree, to which an effective dose of either protein affects the subsequent reaction to the other, can be readily ascertained. By a careful choice of the order in which the doses are given, as much information can often be obtained from one such experiment, with regard to the relative sensitiveness to the different proteins, as would require the injection and reinjection of a long series of animals, if the more usual method of studying anaphylaxis in the guinea-pig were adopted. For purposes of control a few experiments were made by the ordinary method of intravenous reinjection into the intact animal, with observation of the degree of severity of the resulting symptoms as an index of the sensitisation. Young virgin female guinea-pigs were chosen, weighing about 180 g. at the time of the preparatory injection, so that the test of sensitisation, after an interval of two to three weeks, or more, was made when the animals weighed about 250 g. on an average.

The apparatus used was arranged, in all material particulars, similarly to that described by Dale and Laidlaw [1912]. The vessel in which the uterine

horn under experiment was suspended contained, in all cases, 70 cc. of Ringer's solution. The "control" uterine horn was kept, till required, in a tube of the same solution, immersed in the same constant-temperature bath as the experimental vessel. The contents of the experimental vessel, and of the tube containing the control, were oxygenated and stirred by a stream of bubbles. The temperature employed was between 37° and 39° C. The lever recording the uterine contractions magnified twice or thrice, according to the size and activity of the uterus under observation. Similarly the weighting of the lever was varied so as to secure, in each case, efficient extension, without imposing undue resistance to contraction.

The Ringer's solution used was made up according to one or other of the formulae mentioned by one of us in an earlier paper. The results obtained were of the same kind with either, but we gathered the impression that, for keeping the general sensitiveness of the control horn at the same level as that first tested, during a long experiment lasting some hours, the solution called "Ringer I" in the earlier paper was, on the whole, the better. The various proteins tested were made up in Ringer's solution, usually in 1 % solution. The albumin and pseudoglobulin could be dissolved directly in the Ringer's solution without difficulty. Euglobulin so treated was imperfectly soluble. Although the imperfect, opalescent suspension, obtained with Ringer's solution alone, seemed to act perfectly well on the plain muscle when added to the bath, it was considered better, after the first few experiments, to use it in a more highly dispersed condition, in order to obtain a more accurate comparison of its effect with that of pseudoglobulin. With this object, the required amount was weighed out and rubbed up with a little Ringer's solution into a fine emulsion. To this was added 0·1 cc. N/10 NaOH for each 100 mgm. of euglobulin. The relatively clear solution so obtained was then diluted with Ringer's solution to a strength of 1 % euglobulin; the proportion of added soda, after such dilution, corresponding, therefore, to 1/1000 normal. Controls showed that a pure solution of soda, in this strength, when added to the bath in volume corresponding to those of the test doses of euglobulin solution, produced no noteworthy effect on the uterine activity.

A few experiments were also made on anaesthetised dogs, with record of the blood-pressure, to confirm the general applicability of some of the results obtained with guinea-pigs.

Results.

Animals sensitised to whole horse-serum.

We first tested the reaction, to the pure serum proteins, of uterine muscle from guinea-pigs sensitised by a hypodermic injection of 0·01 or 0·1 cc. of whole horse-serum, given two to three weeks previously.

(*a*) *Euglobulin and Pseudoglobulin.* In two cases, out of some eighteen guinea-pigs tested, sensitisation, for some unexplained reason failed, so that the uterine muscle responded but weakly even to large doses of horse-serum. Except in these instances the plain muscle of guinea-pigs, which had received a sensitising injection of whole horse-serum, exhibited a pronounced sensitiveness to the euglobulin and pseudoglobulin of horse-serum. The sensitiveness to the two showed a general parallelism, so that, if one horn of the uterus gave a large contraction in response to 1 mgm. of euglobulin, the second horn usually reacted similarly to 1 mgm. of pseudoglobulin. Similarly, if the first horn tested gave only a small reaction with 1 mgm. of euglobulin, and a subsequent maximal reaction with 10 mgms. of the same, it was usually found that the second horn gave effects of the same order with the same doses of pseudoglobulin.

At first sight, therefore, the results might easily have been interpreted as signifying that the euglobulin and pseudoglobulin were acting as one common antigen. It will be shown later that the effects of sensitising by the separate proteins, instead of by whole serum, exclude such a supposition; but there were clear indications of the distinct antigenic properties of the two globulins, even in the experiments with whole serum. Such indications were obtained by studying the effects of desensitisation. In an earlier paper it was shown that the application of an adequate, effective dose of an antigen, to which the plain muscle has been made anaphylactic, will permanently remove its sensitiveness to that antigen, while leaving it, in other respects, practically normal. The question therefore arises, whether a dose of euglobulin, which renders indifferent to further euglobulin a preparation originally sensitive to both globulins, will at the same time remove its initial sensitiveness to pseudoglobulin, and *vice versâ*. There are three possibilities, and we have found examples of each, in our series of guinea-pigs sensitised by whole serum.

(i) The two globulins may appear to act as a common antigen, so that one horn may respond by a maximal contraction to a certain dose of euglobulin, and be thereafter insensitive not only to euglobulin, but to pseudoglobulin

in like dosage; while the second horn may show an exactly parallel response to, and complete desensitisation by a like dose of pseudoglobulin. This type of reaction is illustrated by the following experimental record, Figs. 1 and 2 reproducing the corresponding tracings.

Guinea-pig No. 9. Sensitised by hypodermic injection of 0·1 cc. of whole horse-serum 16 days previously. Bath-volume 70 cc. Ringer's solution changed after the effect of each dose was complete.

1*st horn of uterus* (Fig. 1).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of euglobulin. Maximal contraction.
10 mgm. of pseudoglobulin. Nil.

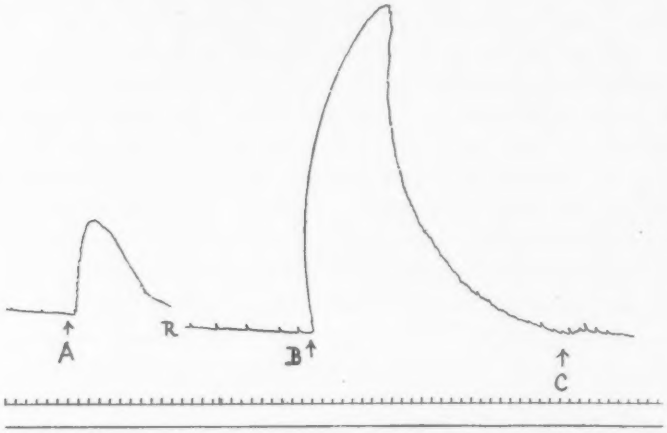

Fig. 1. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 16th day. 1st horn A. 1 mgm. of euglobulin. B. 10 mgm. of euglobulin. C. 10 mgm. of pseudoglobulin. Time marker (in all figures) marks intervals of 30 seconds. R (in all figures) indicates change of Ringer's solution.

2*nd horn of uterus* (Fig. 2).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of pseudoglobulin. Maximal contraction.
10 mgm. of euglobulin. Nil.

It will be seen that a dose of 10 mgm. (1 in 7000) of either euglobulin or pseudoglobulin produced a maximal response of the muscle, after the sub-maximal effect of 1 mgm. of euglobulin had been obtained; but that the maximal response, to 10 mgm. of either, left the organ insensitive to the same dose of the other. Such a result is not uncommon, and seems to be

especially frequent in cases, such as that quoted, in which the sensitiveness is not of a high order. It may be noted that the apparently rather smaller amplitude of contraction in response to 10 mgm. of pseudoglobulin (Fig. 2) as compared with that to 10 mgm. of euglobulin (Fig. 1), is attributable to the use of Ringer II in the former, and Ringer I in the latter case, for the purpose of comparing their suitability for this type of experiment. It is no index, therefore, of preferential sensitisation, which is excluded by the perfect mutual desensitisation.

(ii) There may be sensitisation, to either euglobulin or pseudoglobulin in higher degree than to the other. The difference was in no case very conspicuous, and its demonstration depended chiefly on the phenomena of desensitisation. Thus we might find that one horn responded maximally to

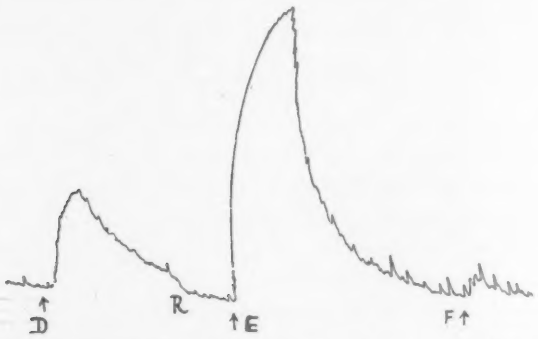

Fig. 2. From same experiment as Fig. 1. 2nd horn. D. 1 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin. F. 10 mgm. of euglobulin.

1 mgm. of euglobulin, gave a second smaller response to 10 mgm. of euglobulin, and was thereafter desensitised not only to euglobulin, but to pseudoglobulin also; while the second horn after practically similar responses to 1 mgm. and 10 mgm. of pseudoglobulin, would be completely insensitive to this protein, but still capable of giving a large response to 10 mgm. of euglobulin. In another case the reverse condition would obtain, the plain muscle desensitised to pseudoglobulin failing to respond to euglobulin, while the second horn, when desensitised to euglobulin, still responded to pseudoglobulin. A couple of examples will suffice to illustrate the point, the importance of which was that it gave us the first indication of an antigenic distinction between the two proteins, which experiments on sensitisation with the separate proteins confirmed.

Experiment 4. Guinea-pig sensitised with 0·1 cc. of horse-serum. 22nd day.

1*st horn.*

1 mgm. euglobulin. Maximal contraction.
5 mgm. euglobulin. Smaller contraction.
5 mgm. euglobulin. Nil.
5 mgm. pseudoglobulin. Nil.

2*nd horn.*

1 mgm. pseudoglobulin. Maximal contraction.
5 mgm. pseudoglobulin. Nil.
5 mgm. pseudoglobulin. Nil.
5 mgm. euglobulin. Large contraction.
5 mgm. euglobulin. Nil.

Experiment 7. Guinea-pig sensitised with 0·1 cc. horse-serum. 21st day.

1*st horn.*

1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin. Large contraction (? Maximal).
1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Nil.
10 mgm. euglobulin. Nil.
10 mgm. pseudoglobulin. Nil.
40 mgm. pseudoglobulin. Nil.

The above two records show preferential sensitisation to euglobulin. In the following the predominant sensitiveness to pseudoglobulin is shown not only in the greater effect of desensitisation to this protein on the subsequent response to euglobulin, but in the lower initial effective dose of the former.

Guinea-pig sensitised with 0·04 cc. antidiphtheritic horse-serum + 1 test dose diphtheria toxin. 24th day.

1st horn.

1 mgm. pseudoglobulin. Full contraction.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Slight contraction.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Good contraction.
10 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Very small contraction.
10 mgm. pseudoglobulin. Large contraction.
10 mgm. pseudoglobulin. Nil.

In this record there is an obvious indication that complete desensitisation to euglobulin is more difficult to achieve than that to pseudoglobulin. This point is clearly discernible in a number of our records. A probably related effect is also to be seen in the above, namely, the tendency of the plain muscle to recover from the desensitisation to euglobulin, and show a revival of response to this protein at a later stage of the experiment. This may indicate a tendency of the euglobulin-antibody complex to dissociate, or it may be attributable to the physical properties of the euglobulin solution, on account of which this antigen less readily reaches and saturates the antibody in the deeper layers of the uterine muscle. We have no direct evidence in favour of either interpretation, but the fact is repeatedly illustrated in our records.

(iii) In some of our records from guinea-pigs sensitised with whole horse-serum, the distinct antigenic properties of the two globulins are made evident by the fact that, while the plain muscle responds with equal readiness to an initial dose of either eu- or pseudoglobulin, desensitisation to either of these leaves a remainder of sensitiveness to the other. This is most readily shown by producing a *relative* desensitisation, by using small doses throughout, as in the following record.

Experiment 12. Guinea-pig sensitised with 0·1 cc. horse-serum. 14th day.

1st horn.

1 mgm. euglobulin. Good contraction.
1 mgm. euglobulin. Small contraction.

1 mgm. euglobulin. Nil.
1 mgm. pseudoglobulin. Fair contraction.
1 mgm. pseudoglobulin. Nil.

2nd horn.

1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
1 mgm. euglobulin. Fair contraction.

It is probable, to judge from the results of other experiments, that the 1st horn, when no longer giving a perceptible response to 1 mgm. of euglobulin, would have responded to 10 mgm.; and similarly with pseudoglobulin in the case of the second horn. The point to be emphasised is that, in either case, the muscle, when reduced to a condition in which it fails to respond at all to 1 mgm. of one of the globulins, still shows a definite sensitiveness to the same dose of the other.

(*b*) *Albumin.* Our earlier results seemed to support the finding of Doerr and Russ, that serum albumin did not act as an anaphylactic antigen. In a series of preparations from guinea-pigs sensitised to whole serum, we found that the plain muscle seemed completely indifferent to a dose of 5 or 10 mgm. of the albumin, subsequently giving a maximal response to 1 mgm. of either globulin. The result seemed so definite, and was obtained with such regularity in the early series, that for some time we abandoned experiments with albumin, considering its inefficacy as settled. At a later stage of the investigation six guinea pigs received the usual sensitising injection of 0·1 cc. of horse-serum, to test another point, and it happened that the uterus was tested with 1 mgm. of pure serum albumin, which produced a typical, nearly maximal, response. The uteri of the other guinea-pigs of this series were then tested with albumin, and all showed a similar high degree of sensitiveness to this protein. The difference could not be attributed to a deficiently purified albumin, since the preparation which now proved an effective stimulus was the same as that which had previously proved ineffective; and the highly purified sample obtained by recrystallising four times, as described above, was quite as effective a stimulant as that purified by the precipitation method only. The only difference between the two series was in the time elapsing between the sensitising injection and the performance of the test. In the earlier series, in which no sensitiveness to albumin was detected, the interval was two to three weeks; in the later series, in which a good reaction to albumin was

observed, it was upwards of thirty days—four to five weeks. A further series of six guinea-pigs was therefore prepared, being sensitised with 0·1 cc. horse-serum and kept for twenty-nine to thirty days before testing, with the same result, that after this interval a sensitiveness of the plain muscle to albumin had developed.

There was some indication that, concurrently with the late development of the sensitiveness to albumin, the sensitiveness of the plain muscle to the globulins, which attains its maximum somewhere between the 14th and 21st days, undergoes some decline, to judge by the size of the initial dose needed to produce a pronounced reaction. The following record illustrates the point.

Fig. 3. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 31st day. 1st horn. A. 1 mgm. of albumin.

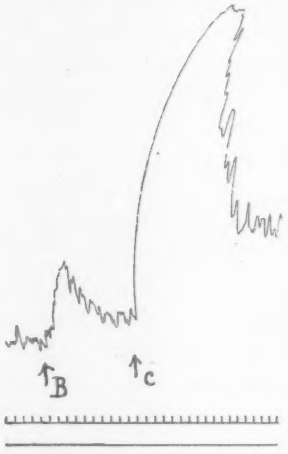

Fig. 4. Same experiment as Fig. 3. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

Experiment 26. Guinea-pig sensitised with 0·1 cc. horse-serum. 31st day.

1*st horn.*

1 mgm. albumin.	Maximal contraction (see Fig. 3).
10 mgm. albumin.	Weak contraction.
10 mgm. albumin.	Nil.
1 mgm. euglobulin.	Nil.
10 mgm. euglobulin.	Moderate contraction.

2nd horn.

1 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Maximal contraction (see Fig. 4).
10 mgm. euglobulin. Nil.
10 mgm. albumin. Nil.

It will be noticed that though the plain muscle, in this case, showed a higher sensitiveness to albumin than to euglobulin, as judged by its response to a small dose (1 mgm.) of either, it is more readily desensitised to albumin by an effective dose of euglobulin, than to euglobulin by albumin.

The results obtained, by observing the response of isolated plain muscle, suggested that the failure of previous workers to observe an anaphylactic reaction in the serum-sensitive guinea-pig, when pure albumin was used for the reinjection, might have been due to the allowance of an insufficient incubation-period for the development of sensitiveness to this protein. It seemed desirable, therefore, to confirm, by experiments on the intact animal, the fact that true anaphylaxis to serum albumin does occur, though late in its development; in other words, to ensure that the reaction of the isolated plain muscle, *in vitro*, gives, in this case also, a genuine indication of the occurrence of the anaphylactic condition, as seen in the whole animal. A guinea-pig from the same series as that used in Experiment 26, and similarly sensitised by a hypodermic injection of 0·1 cc. of horse-serum, was therefore tested on the 32nd day after sensitisation. The albumin was made up for the reinjection in 1 % solution in physiological saline. Of this solution 1 cc. (10 mgm. of albumin) was injected into the jugular vein, exposed by a small incision under local cocaine-anaesthesia. Symptoms appeared immediately after the completion of the injection, and typical anaphylactic death followed in about 3 minutes. The post-mortem appearances were quite characteristic—fixed distension of the lungs, and retarded coagulation of the blood.

It seemed desirable to confirm, in another species of animal, this efficacy of serum-albumin as an anaphylactic antigen, provided the incubation period is sufficiently long. A dog therefore received a sensitising injection of 5 cc. of horse-serum hypodermically. On the 28th day after this injection the dog was anaesthetised by A.C.E. mixture, after a preliminary injection of morphia. Arrangements were made for recording the blood-pressure from the carotid artery, and injection into the femoral vein. The record was started, and 20 cc. of a 5 % solution in saline of the pure albumin of horse-serum were then run slowly into the vein. A profound and typical shock

occurred, the blood-pressure commencing to fall rapidly during the injection, continuing to fall after the injection was completed, and remaining long at a very low level. The fall of pressure was accompanied by the typical dyspnoea, and a sample of blood, obtained while the depression persisted from the other carotid artery, showed no sign of clotting after standing for 24 hours.

Guinea-pigs sensitised to individual proteins.

(*a*) *Sensitised to euglobulin.* Sensitisation with euglobulin produced a more clearly specific sensitiveness than that with either of the other proteins. When a guinea-pig was killed 13 to 17 days after a preliminary injection of 1 mgm. of euglobulin, its uterine plain muscle responded typically to 1 mgm. or 10 mgm. of euglobulin, but was apparently indifferent either to albumin or pseudoglobulin, at any rate within the limits of dosage employed in these experiments (see Figs. 5 and 6).

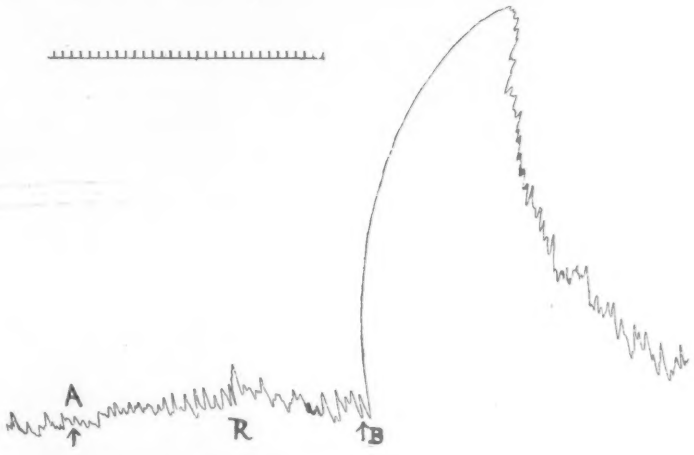

Fig. 5. Guinea-pig sensitised with 1 mgm. of euglobulin. 17th day. A. 5 mgm. of albumin. B. 1 mgm. of euglobulin.

(*b*) *Sensitised to pseudoglobulin.* The available samples of pseudoglobulin produced a sensitisation to either pseudoglobulin or euglobulin, but the sensitiveness to the former was always definitely superior, as the following record shows.

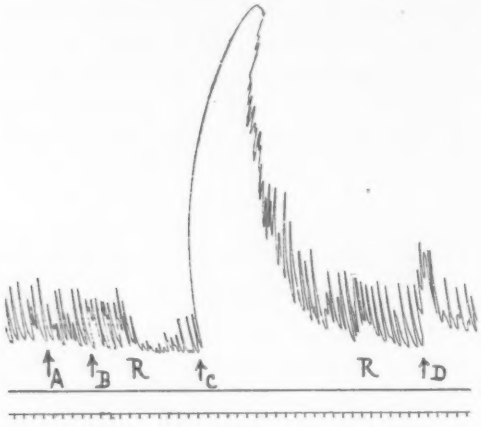

Fig. 6. Guinea-pig sensitised with 1 mgm. of euglobulin. 13th day. A. 1 mgm. of pseudoglobulin. B. 10 mgm. of pseudoglobulin. C. 10 mgm. of euglobulin. D. 10 mgm. of euglobulin.

Experiment 30. (See Figs. 7, 8 and 9.) Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day.

1*st horn.*

1 mgm. pseudoglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Good but submaximal contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Nil.
10 mgm. pseudoglobulin. Good but submaximal contraction.

It is clear that 1 mgm. pseudoglobulin, as a first dose, is much more effective than 1 mgm. euglobulin under the same conditions; further that complete desensitisation to euglobulin weakens, but by no means annuls the sensitiveness to pseudoglobulin.

Experiment 35. Guinea-pig sensitised with 0·1 mgm. pseudoglobulin 18 days.

1*st horn.*

0·1 mgm. pseudoglobulin. Small contraction.
1 mgm. pseudoglobulin. Moderate contraction.
1 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Very small contraction.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Maximal contraction.
10 mgm. euglobulin. Nil.
1 mgm. pseudoglobulin. Small contraction.

Fig. 7. Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day. 1st horn. A. 1 mgm. of pseudoglobulin.

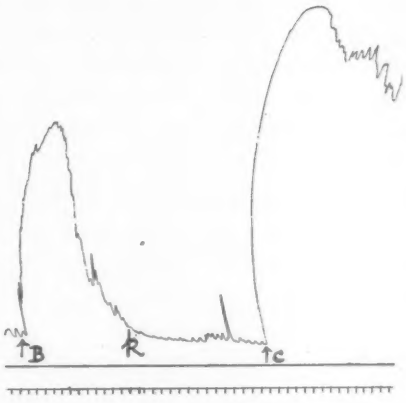

Fig. 8. Same experiment as Fig. 7. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

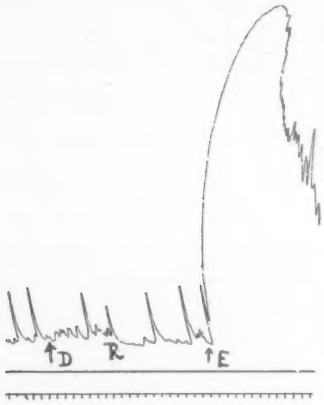

Fig. 9. Continuation of Fig. 8. D. 10 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin.

In this experiment an error was made in dilution, which resulted in the dose of pseudoglobulin being throughout only one-tenth of what had been intended. The mistake, however, was not without advantage, in bringing out very clearly the preponderance of sensitiveness to pseudoglobulin. For the result showed that desensitisation to 1 mgm. of pseudoglobulin almost abolished the response to 10 mgm. of euglobulin; while after desensitisation to 10 mgm. of euglobulin, the muscle responded definitely, though not powerfully, to 1 mgm. of pseudoglobulin. It may also be noted that, whereas the first horn gave a small, but quite definite response even to 0·1 mgm. pseudoglobulin—*i.e.* to a dilution in the testing-bath of 1 in 700,000—the second horn gave no response at all to 1 mgm. (1 in 70,000) of euglobulin, but was stimulated to maximal contraction by 10 mgm. (1 in 7,000).

Sensitised to serum albumin.

All the guinea-pigs used in these tests received a sensitising hypodermic injection of 1 mgm. of purified serum albumin. Unfortunately lack of time, and the exhaustion of our small stock of purified pseudoglobulin, prevented any discriminating tests between the antigenic properties of pseudoglobulin and albumin, which would have given information of some interest. We were able, however, to show, more definitely than with the guinea-pigs sensitised to whole serum, the antigenic independence of albumin and euglobulin. Few experiments were made with isolated muscle, and it will be sufficient to quote one. It will be noted, both in this case and in the records of experiments on the intact animal, that when pure albumin is used for sensitisation, anaphylaxis to this protein, though still somewhat slowly developed, appears at an earlier stage than when the immunity reaction is complicated by the presence of the other serum proteins in the sensitising inoculum.

Experiment 35. Guinea-pig sensitised with 1 mgm. serum albumin hypodermically. 22nd day.

1*st horn.*

1 mgm. serum albumin. Good, but not maximal contraction.
10 mgm. serum albumin. Nil.
10 mgm. euglobulin. Nil.

2*nd horn.*

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Trace of response (doubtfully specific).
1 mgm. albumin. Good, but not maximal contraction.

For the experiments on anaphylaxis *in vivo* two guinea-pigs (*A* and *B*) were first sensitised, each with 1 mgm. albumin as usual. Twenty-two days later, when each weighed 250 g. *A* was tested by intravenous injection of 10 mgm. albumin in 1 cc. of saline. This caused pronounced symptoms—coughing, chattering, erection of hair, falling on the side. After about 20 minutes, however, the animal was obviously recovering, and 10 minutes later was practically normal again. Next day it was perfectly well.

On the 35th day both guinea-pigs weighed about 275 g. *B* received 5 mgm. of albumin in 0·5 cc. of saline intravenously, showed immediate pronounced symptoms, and died within 2 minutes.

A, which had survived the injection on the 22nd day, was given a second injection of 10 mgm. on the 35th day. Symptoms developed slightly more slowly than in *B*, but death occurred within 3 minutes.

Both animals showed the typical post-mortem picture of acute anaphylaxis—lungs fixed in distension, slowly clotting blood, etc.

A further series of twelve guinea-pigs (numbered I–XII) were sensitised with 1 mgm. of albumin and examined as follows.

No. of guinea-pig	Weight in g.	No. of days after sensitisation	Dose given intravenously	Result
I	275	10	Albumin 20 mgm.	Slight symptoms, recovered
II	275	10	„ 10 „	Nil
III	275	17	„ 20 „	Death in 3 mins.
IV	250	17	„ 10 „	„ 4 „
V	250	17	„ 5 „	„ 5 „
VI	225	17	„ 2 „	„ 3 „
VII	215	17	„ 1 „	„ 3 „
X	220	27	Euglobulin 5 mgm.	Nil
XII	210	27	Albumin 0·5 mgm.	Death in 2 mins.
X (2nd injection)	220	28	„ 1 mgm.	„ 4 „
I (2nd injection)	300	30	Albumin 10 mgm.	Nil
II (2nd injection)	300	30	„ 1 „	Nil

Guinea-pig VIII was used for the isolated muscle test quoted above; guinea-pigs IX and XI died from accidental causes before testing.

There are several points worth noting in the above table. The sensitiveness to albumin is practically non-existent on the 10th day, by which sensitiveness to whole serum is well developed in animals previously injected with this. By the 17th day, however, in this series a very high sensitiveness to albumin has appeared, the lower limit of fatal dosage for guinea-pigs of this size not being reached in the experiment. The experiments on the 27th and 28th days show that there is no reaction to euglobulin in these

guinea-pigs, even in a dose at least ten times as large as the fatal dose of albumin; nor does the injection of euglobulin notably modify the effect of albumin given on the next day. Lastly, it is seen that, when large injections have been given on the 10th day, *i.e.* before sensitiveness to albumin has appeared, no sensitiveness of the animal to albumin can be detected by intravenous injections given on the 30th day. It has been shown by one of us that such lack of apparent sensitiveness may be due either to genuine desensitisation of the tissues, or to an excess of antibody in the circulating fluids, protecting from the antigen tissues which, when isolated, are highly sensitive. It must remain an open question, for the present, which of these two conditions accounted for the lack of intoxication in this instance.

Note on Anaphylaxis to Pure Egg-albumins.

The production in the guinea-pig of a high degree of anaphylaxis to the pure, crystallised albumin of the hen's egg has been previously described by Armit [1910] and by Wells [1908]. Wells [1911] has also shown that ovovitellin, from egg-yolk, acted as a quite separate antigen from the proteins of egg-white[1]. In the preceding sections we have given evidence of the separate action, as anaphylactic antigens, of the different proteins of one serum. By way of contrast it seems appropriate to make preliminary mention here of some experiments made by one of us (H. H. D.) on anaphylaxis to the pure crystalline albumins from the egg-white of hens and ducks, in the preparation of which Dr A. J. Ewins kindly co-operated. The albumins were crystallised by the familiar method of Hopkins [1899], three successive crystallisations being given to each sample. The eggs were obtained from a private source, and there can be no question of any mistake in specific identification. The albumin from the ducks' eggs was rather more difficult to crystallise than that from hens, a larger addition of acid being required to initiate the first crystallisation. When once obtained in clean crystals, however, it was recrystallised as readily as that of the hen. The final crop of crystals, in both cases, was dialysed free from ammonium sulphate, and the watery solutions were evaporated to dry scale-preparations at room-temperature *in vacuo*, solutions being then prepared as required. Female

[1] There is a somewhat extensive literature on the crossed specificity of different egg-whites, which has not been dealt with here, since the experiments made by us at present have been concerned solely with the crystalline albumins.

guinea-pigs received injections of 0·1–1 mgm. of either albumin, and the isolated uterine plain muscle was tested for sensitiveness after three weeks. The result can be stated in a line. There was no evidence of any antigenic distinction between the two albumins. The plain muscle of guinea-pigs, which had been injected with hen's albumin was quite as sensitive to duck's albumin as to hen's, and the same was true of preparations from guinea-pigs injected with duck's albumin. Moreover an effective dose of either albumin

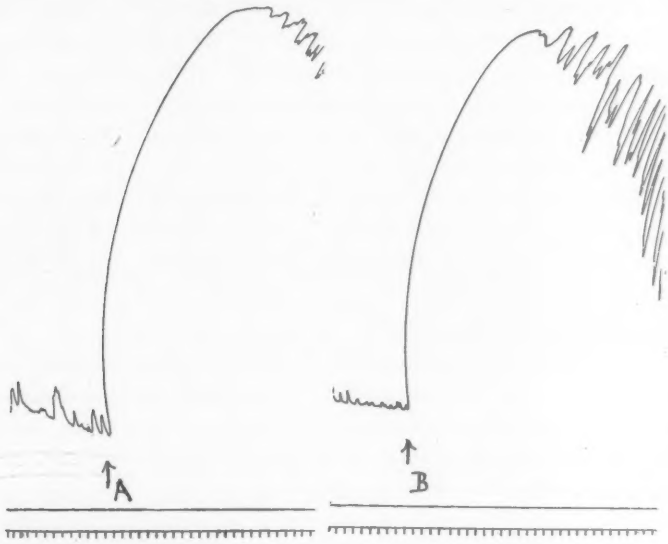

Fig. 10. Guinea-pig sensitised with 1 mgm. of crystalline albumin of hen's egg-white. 22nd day. A. 1st horn. Effect of 0·05 mgm. duck's egg-albumin. B. 2nd horn. Effect of 0·05 mgm. hen's egg-albumin.

was followed by complete insensitiveness to the other, irrespective of which had been used as the sensitising dose. If the notebook record had been lost, it would have been impossible, from the response of the plain muscle, to form any opinion as to which albumin had been used for sensitisation. Fig. 10 illustrates the corresponding reactions of the two uterine horns from a guinea-pig sensitised with hen's albumin. It may be added that neither preparation had any effect on uterine muscle from normal, unsensitised guinea-pigs.

Summary and Discussion.

The following are the main conclusions to be drawn from the above experiments:

1. Each of the three proteins separable from horse-serum by their physical and chemical properties—euglobulin, pseudoglobulin, albumin—can act as an anaphylactic antigen.

2. A guinea-pig which has received a sensitising injection of one of these proteins is more sensitive to that one than to either of the others from the same serum. In some cases the sensitisation seems to be rigidly "specific," as when euglobulin produces sensitiveness to euglobulin but not to pseudoglobulin, or when albumin produces a high degree of sensitiveness to albumin but none to euglobulin.

3. The sensitiveness of the guinea-pig to albumin is later in development than that to the globulins; this difference possibly accounts for the fact that previous authors failed to detect the sensitising property of albumin. The difference is especially marked when sensitisation is carried out with whole serum, containing all three.

4. An effective dose of any of the proteins, to which the guinea-pig's plain muscle has been sensitised, partially or completely desensitises it to the other proteins of the same serum.

5. The crystalline albumins from the white of the eggs of hens and ducks show no distinction of antigenic properties.

The effects of "crossed" desensitisation, in particular, may give the impression that there is a considerable community of antigenic function among the proteins of one serum. Such an impression, however, is not completely justified, since one of us (H. H. D.) has previously shown that, when a guinea-pig is simultaneously sensitised to two or more antigens of widely different origin, and of undoubted antigenic independence—*e.g.* egg-white and horse-serum—desensitisation of the plain muscle to one may greatly weaken the sensitiveness to the other. On the other hand, the fact that sensitisation with one of the globulins frequently produces a smaller, but quite definite sensitiveness to the other, shows that, at any rate, the preparations of these two substances at our disposal were not completely independent in their antigenic properties. But the question necessarily arises whether this overlap is a genuine phenomenon, or is due to the fact that, even in such elaborately purified preparations as those which we used, the separation was

not ideally perfect. This possibility is worthy of consideration, in that the amount of a protein needed to evoke some degree of sensitiveness is almost inconceivably small. Wells [1908], for example, succeeded in sensitising guinea-pigs with 1/20,000 milligram of egg-albumin. This explanation of the overlap in sensitisation is favoured by the evidence that it appears most prominently when the impurity of the sensitising protein is most likely to be considerable. The purification of euglobulin is facilitated by its insolubility in weakly acidulated water. We have found that a rigidly specific sensitiveness to euglobulin occurs comparatively frequently. On the other hand, we never succeeded, by injection of pseudoglobulin in producing a sensitiveness to pseudoglobulin alone; sensitiveness to euglobulin in addition was always detectable, though it might be relatively weak, especially when the sensitising dose was small. But the purification of pseudoglobulin from the last traces of euglobulin is so difficult, that it may be doubted whether it is possible by existing methods. It is similarly difficult to make a sharp separation between pseudoglobulin and albumin, and we think it probable that a similar overlap of sensitisation would have been detected between these two proteins. Unfortunately the material was exhausted before this point had been put to the test. Again, if we regard the overlap of antigenic properties as due to incomplete separation, it would be expected that the sharpest distinction would be observed between euglobulin and albumin, each of which can be obtained with tolerable certainty uncontaminated by the other. The results were quite in accordance with this expectation, since the plain muscle from a guinea-pig sensitised with euglobulin seemed quite indifferent to albumin, and *vice versâ*. On the whole, then, the evidence seems to us in favour of the view that the different proteins of a serum, in so far as they can be obtained pure from one another, act as independent anaphylactic antigens.

This narrowing down of immunological "specificity," to a discrimination between the different pure proteins of a single tissue of one species, is by no means new. Wells [1911], as above mentioned, found evidence of complete antigenic independence between the "ovovitellin" of egg-yolk, on the one hand, and the proteins of egg-white on the other; he found, however, a considerable overlap in the antigenic properties of the albumin and globulin of egg-white, however carefully they were purified. Ovomucoid was again an independent antigen. Wells and Osborne [1911, 1913] have furnished numerous instances in which the different pure proteins from one plant species act as separate antigens, while the corresponding pure proteins from allied species have common antigenic properties. Such evidence gives to the

fundamental problem of immunity a more hopeful aspect, by its suggestion that the antigenic distinction is possibly dependent on such properties of the different proteins as may be open to the ordinary methods of chemical and physical enquiry, and not necessarily on some biological difference of so mysterious a nature, as to elude investigation by such methods. Our own evidence of the antigenic independence of the proteins from one blood-serum, and of the community of antigenic function between the pure albumins of egg-white from species so widely separated as the fowl and the duck, has the same tendency. It seems clear to us that every sensitisation with a whole serum, for example, is in reality a complex multisensitisation; and that it is essential to work with individual pure proteins, in any attempt to investigate the nature of the difference, of which antigenic independence is an expression.

The question has often been debated whether the difference is a chemical or a physical one, without giving these terms any greater precision of application. One possibility of chemical difference we are in a position to eliminate. An analysis of the separated proteins from horse-serum, used by us in these experiments, had been carried out by one of us [P. Hartley, 1914] by the aid of van Slyke's method. It was found that albumin, as others had shown, differed from the globulins in yielding, on hydrolysis, a higher proportion of the diamino-acids, and in containing a higher proportion of cystine. It was further shown that, of the diamino-acids, the excess yielded by albumin, as compared with the globulins, was greatest in the case of lysine, which albumin contained in about a 50 % higher proportion. No such difference was found between the two globulins, which, as regards the distribution of their nitrogen among the different amino-acids, showed no difference of any note. There is no help to be obtained, therefore, in this direction towards an explanation of the antigenic difference between the globulins. It is clear, however, that there is abundant room for difference of chemical structure even between protein molecules built up from the same amino-acids in identical proportions. Indeed, if the highly specific combining properties, which give a protein molecule its antigenic character, are related to chemical structure at all, it seems likely that they would be as much dependent on the pattern of the structure of the molecule, on the order in which the amino-acids are linked up in it, as on their nature and the proportions in which they are present. A relation between the stereo-chemical and antigenic characters of the protein molecule is, indeed, definitely suggested by the observation of Ten Broek [1914], who found that a protein racemised by Kossel's method had lost all

power of acting as an antigen, just as Dakin and Dudley [1913] found that it had lost its susceptibility to the action of proteolytic enzymes. Dakin [1912] has given reason for believing that the amino-acids which escape racemisation in this process, and are therefore obtained in the natural, optically active condition on subsequent acid hydrolysis of the racemised protein, are those occupying the terminal positions of the peptide chains out of which the protein molecule is built. Dudley and Woodman [1915], using Dakin's method, found evidence of a structural difference of the kind under discussion between the caseinogens of cow's and of sheep's milk—proteins consisting of identical amino-acids in identical proportions. In the case of sheep's caseinogen the tyrosine wholly and the lysine largely escaped racemisation, while they were both completely racemised in that of cow's caseinogen. The instance is an unsatisfactory one for our present purpose, since the antigenic properties of caseinogen are relatively feeble, and the caseinogens from different species show no clear disparity of antigenic properties. In the only other case of proteins with identical amino-acid content which have hitherto been thus compared, the euglobulin and pseudoglobulin of colostrum, there is presumptive evidence of an antigenic difference of the same kind as that which we find existing between the corresponding serum-proteins. In this instance Dudley and Woodman detected no difference by the racemisation method. So that, so far as evidence of this kind has been obtained at present, it fails to support, and might even seem to discredit the view that antigenic properties depend on the structural pattern of the protein molecule. We have an example of structural difference, without antigenic disparity, and another, with no structural difference detected, in which some degree of antigenic independence may be presumed. It must be remembered, however, that the method reveals only one kind of structural difference, the effect of which on the antigenic properties might well be subsidiary to other differences, which the method fails to detect. The observation that two similar proteins from different species, the caseinogens of the cow and sheep, while as yet indistinguishable by other methods, show a definite difference in the order of amino-acid linkage, remains highly significant, and seems to indicate the lines along which the possibility of a connexion between antigenic properties and chemical structure may yet be investigated.

It has not been assumed, in thus considering the possibility of a chemical basis for antigenic specificity, that the combination between antigen and antibody is of the type of an ordinary chemical union. There is, of course, much evidence indicating that the association has the character rather of

an adsorption. This, however, by no means precludes the possibility that it may be conditioned ultimately by the structure of the antigenic molecule. There is similarly strong evidence in favour of regarding the association between an enzyme and its substrate as an adsorption; yet it is essentially dependent on the configuration of the substrate molecule. Again, Barger and his co-workers [1915] have demonstrated the connexion between the formation of coloured adsorption-compounds with iodine and a certain type of structure in organic compounds. They also showed, however, the dependence of the reaction on surface-phenomena, in that a colloidal solution, or suspension of the substance in amorphous aggregates, was necessary for the formation of the typical coloured complex with iodine. It is not improbable that the antigenic behaviour of a protein is similarly conditioned by its state of dispersion. Miss Chick [1914] and others have given reason for considering euglobulin as possibly formed by association of pseudoglobulin with a lipoid, which gives to euglobulin its phosphorus content and its characteristic physical properties. It is conceivable that the antigenic difference between a pseudoglobulin and the euglobulin from the same serum may be due to the peculiar physical characteristics conferred on the euglobulin by associated lipoid. We hope later to test this possibility by comparing the antigenic action of a pseudoglobulin, with that of an artificial "euglobulin" made from it. But even if that were the case, such characteristics could hardly be more than one factor in the phenomenon of specificity; and to form an idea of its significance, it would be necessary to examine the degree of antigenic independence exhibited by pure euglobulins from the sera of different species. Using the purest proteins obtainable as antigenic units, it should eventually be possible to compare different proteins from the same species, and similar proteins from different species, in respect of their molecular configuration and also of their physical properties, and to trace the relation of each type of character to the antigenic individuality of the substances examined.

A word may be added on some practical bearings of our results. Since all the proteins of a serum can act as anaphylactic antigens, it is evident that the ideal to be aimed at, in concentrating the curative element in a specific immune serum, is simply the reduction of the ratio of total protein to antitoxic value; for the purpose of reducing serum-reactions, the elimination of albumin seems to be as important as that of euglobulin, when the pseudoglobulin is the fraction carrying the therapeutic power. Our observation of the relatively long latent period of the sensitiveness to albumin is also suggestive in connexion with the successive crops of serum rash, which have

been recorded in certain patients as the result of one injection of a serum. It has been suggested [Goodall, 1907] that these represent separate reactions to the sera of the different horses which may have contributed to the serum. It seems to us more reasonable to suppose that they represent the successive appearances, at different time intervals, of sensitiveness to the different serum-proteins.

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XXXV. A COMPARATIVE STUDY OF THE PROTEINS OF THE COLOSTRUM AND MILK OF THE COW AND THEIR RELATIONS TO SERUM PROTEINS.

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(*Received August 1st, 1916.*)

Very diverse views have been propounded from time to time as to the nature of the protein constituents of cow's milk. On the one hand Duclaux and others have asserted that only one protein, caseinogen, is present, existing in coagulable and non-coagulable forms. On the other hand the literature of milk proteins includes claims to the isolation of some nine or ten distinct proteins. There can be little doubt that the majority of these latter preparations were of very doubtful purity, and from a close survey of the literature it is clear that the presence of only two distinct proteins has been demonstrated with reasonable certainty, viz. caseinogen and lactalbumin. In normal cow's milk of average quality these are present to the extent of about 3·0 % and 0·4 % respectively. A third protein, with the general characteristics of a globulin, was isolated by Sebelien [1885] and independently by Emmerling [1888], the amount present in milk being stated to be not more than about 0·005 %. More recently Halliburton [1890] has denied the existence of this lactoglobulin, the material isolated by Sebelien being regarded by him as lactalbumin.

There can be no such doubt as to the existence of a globulin in the colostrum—the fluid secreted in the earliest stages after parturition. The secretion at this stage is characterised by an extraordinary richness in protein material, this forming quite commonly 18 to 20 % of the total secretion. The proportions of caseinogen (4–5 %) and of albumin (0·7–1·5 %) are appreciably higher than in the normal milk of later stages of lactation, but

the great bulk (6–12 %) of the colostrum protein consists of a globulin. With the development of mammary activity this preponderance of globulin rapidly vanishes, and usually within 48 hours of calving the proportion of globulin in the secretion has become similar to, or less than that of albumin, the total protein being correspondingly reduced.

These changes with advance of lactation in the proportions of the three proteins present are well illustrated by the following averages obtained by us with seven cows of the herd at Manor Farm, Garforth (Experimental Farm of the University of Leeds and the Yorkshire Council for Agricultural Education). The cows were milked twice daily after calving.

		Nitrogen present			
	Total %	as caseinogen %	as albumin %	as globulin %	as non-protein %
1st milking	2·40	0·75	0·14	1·32	0·19
2nd „	2·01	0·68	0·17	1·02	0·14
3rd „	1·44	0·59	0·14	0·59	0·12
4th „	0·97	0·51	0·11	0·31	0·04
5th „	0·76	0·46	0·07	0·20	0·03
6th „	0·75	0·46	0·06	0·20	0·03
7th „	0·69	0·42	0·06	0·18	0·03
8th „	0·65	0·46	0·05	0·12	0·02

A more prolonged series of analyses made by Sutherst [1902] with a single cow gave very similar results.

It has been commonly assumed that the protein preparations obtained from colostrum are identical with the similarly named preparations obtainable by the same methods from milk, but little evidence has yet been adduced in justification of this assumption. Even in the case of caseinogen the existing evidence amounts to little more than the close agreement in physical properties and ultimate chemical composition.

In the work described in the present communication we have endeavoured to obtain really conclusive evidence on this point by a study of the chief products of hydrolysis of preparations of caseinogen, globulin and albumin isolated with the greatest care from the colostrum and later from the normal milk of the same cows. Two cows (2AI and 10AI) of the dairy herd at the Manor Farm, Garforth, were used for the purpose, the colostrum being taken in each case immediately after calving, whilst three months later a quantity of the milk of each cow was worked up in precisely similar fashion.

The methods adopted for the isolation and purification of the different

proteins are described fully later. For the characterisation of the proteins the method of analysis elaborated by van Slyke [1911] was used. This method has now become widely known and it will suffice here to indicate that it consists of a quantitative study of the distribution of the nitrogen of the protein between the different nitrogenous ingredients of the mixture obtained by acid hydrolysis of the protein. The determinations made lead finally to a classification of the nitrogen distribution under the following heads:

Ammonia-N, melanin- (or humin-) N, cystine-N, arginine-N, histidine-N, lysine-N, and nitrogen in forms not precipitable by phosphotungstic acid (subdivided into mono-amino-N and non-amino-N).

The method requires only 3–4 g. of protein, and with due allowance for experimental errors, all the nitrogen can be accounted for. The limits of accuracy which must be kept in mind in discussing the results may be gauged from the following data given by van Slyke [1911] which represent as large differences between duplicates as are normally to be expected.

	%		%
Ammonia-N	0·37	Lysine-N	1·23
Melanin-N	0·39	Total N of filtrate ...	1·50
Cystine-N	0·11	Amino-N of filtrate ...	1·10
Arginine-N	1·27	Non-amino-N of filtrate	1·20
Histidine-N	0·93		

It should be remarked that van Slyke's analyses, from which the foregoing table of errors is deduced, were done on duplicate portions of the same hydrolysis solution. In the experiments described in this paper, however, each protein was hydrolysed separately, and thus any errors incurred during the preparation of the proteins or during their hydrolysis would be added to the experimental errors described above.

In order to ensure the strictest possible comparability of the results throughout, the whole of the experimental work has been carried out by one of us (H. R.) and every effort has been made to secure the maximum of accuracy of which the method is capable.

The determination of the histidine-N, lysine-N and non-amino-N by difference rather than by direct estimation would seem to make these figures liable to considerable errors, but experience, as indicated above, shows that the errors are, in actual fact, relatively small [cf. van Slyke, 1911].

In addition to furnishing evidence as to the identity or otherwise of the corresponding proteins of colostrum and milk, our results, taken in conjunction with those obtained by the same methods by Hartley [1914] with the globulins and albumin of ox-serum, enable conclusions to be drawn as

to the identity or otherwise of the globulins and albumin of colostrum and milk with the analogous proteins of ox-serum.

Further, since we have obtained lactoglobulin in pseudoglobulin and eu-globulin forms we have been able to compare the chemical structure of the protein in these two forms.

Caseinogen.

Evidence of the identity of caseinogen prepared from colostrum with that prepared from milk was first adduced by Sebelien [1885] and later by Winterstein and Strickler [1906]. In each case the evidence amounted to little more than the close agreement in ultimate chemical composition, and the similarity of response to familiar qualitative protein tests. Their conclusions have been substantiated, however, by the more recent work of Bauer and Engel [1911] and others in which the precipitin and other biological tests have been used.

Our own results are summarised in Table I, which gives the results of analyses of two preparations each from colostrum and milk.

TABLE I.

Summary of the Corrected Analyses of Different Preparations of Caseinogen in Percentages of the Total Nitrogen.

	From colostrum		From milk		Average		
	(1) Cow 2AI %	(2) Cow 10AI %	(3) Cow 2AI %	(4) Cow 10AI %	Colostrum (1 and 2) %	Milk (3 and 4) %	Average of all four samples %
Ammonia-N	10·42	10·07	10·15	10·35	10·25	10·25	10·25
Melanin-N	1·29	1·35	1·27	1·12	1·32	1·20	1·26
Total N of bases	25·94	26·76	27·52	26·28	26·35	26·90	26·62
Cystine-N	1·34	1·40	1·23	1·24	1·37	1·24	1·30
Arginine-N	8·95	9·87	9·73	8·71	9·41	9·22	9·31
Histidine-N	6·45	6·12	7·17	6·47	6·29	6·82	6·55
Lysine-N	9·20	9·37	9·39	9·86	9·29	9·62	9·46
Total N of filtrate	63·30	62·25	61·00	62·70	62·78	61·85	62·31
Amino-N of filtrate	56·90	55·34	54·06	55·46	56·12	54·76	55·44
Non-amino-N of filtrate	6·40	6·91	6·94	7·24	6·66	7·09	6·87
Sum	100·95	100·43	99·94	100·45	100·69	100·19	100·44

The differences between the results for the four samples are in no case greater than would normally be expected between duplicate analyses of any one preparation of caseinogen, so that one may reasonably conclude that, so

far as the protein part of the molecule is concerned, the caseinogen of colostrum is identical with that of milk.

Globulin.

Hammarsten seems to have been the first to suggest the presence of a globulin in milk, and a substance with globulin-like characteristics was indeed isolated from milk by Sebelien [1885]. He expressed a doubt, however, whether this substance could be regarded as a true globulin.

Halliburton [1890] on repeating Sebelien's work obtained a small quantity of a protein preparation, which, however, he maintained was not a globulin but merely lactalbumin. As a result of further investigation he arrived at the conclusion that milk does not contain a globulin.

There can be no doubt that this conclusion is erroneous. Not only do biological tests point clearly to the presence of a globulin in milk, but we have succeeded in obtaining from milk small quantities of material which, as the results in Table II indicate, is in every way identical with the undoubted globulin prepared from colostrum.

In the case of the preparation from colostrum the total globulin was resolved into pseudoglobulin and euglobulin by dialysis, but owing to the small quantities involved this was not feasible in the case of the preparation from milk.

TABLE II. *Summary of the of Globulin in Per-*

	From colostrum			
	Cow 2AI		Cow 10AI	
	(5) Eu globulin %	(6) Pseudo-globulin %	(7) Eu-globulin %	(8) Pseudo-globulin %
Ammonia-N	7·28	7·19	6·92	7·33
Melanin-N	2·00	2·03	2·40	2·20
Total N of bases ...	24·95	24·57	24·52	23·90
Cystine-N	2·00	1·83	2·04	1·81
Arginine-N	10·93	10·70	10·69	10·41
Histidine-N	3·87	4·16	3·68	3·25
Lysine-N	8·15	7·88	8·11	8·43
Total N of filtrate ...	65·31	65·82	63·97	64·57
Amino-N of filtrate ...	64·46	64·49	63·08	63·31
Non-amino-N of filtrate	0·85	1·33	0·89	1·26
Sum	99·54	99·61	97·81	98·00

From Table II it is clear that the total globulin of milk is identical in composition with that of colostrum. Further, it is obvious that, so far as the protein part of their molecules is concerned, eu-lactoglobulin and pseudo-lactoglobulin are identical. This is in complete accord with the similar observations of Hartley [1914] on euglobulin and pseudoglobulin from ox-serum, and lends further support to the hypothesis of Chick [1914] that euglobulins are protein-lipoid complexes resulting from the interaction and mutual precipitation of the colloidal solution of pseudoglobulin with a lipoid emulsion. That differences do exist between the pseudoglobulin and euglobulin prepared from ox-serum is clear from the work of Hardy [1905] and Haslam [1913], but Hartley's results show that this difference is limited to the non-protein ingredients of the preparations, as do our results for the parallel, if not identical, case of the lactoglobulin preparations.

Albumin.

The results of analysis of three samples of albumin from colostrum and two samples from milk are summarised in Table III.

They show clearly that the lactalbumin of colostrum is identical with that of milk, and thus substantiate the indications of biological tests.

Corrected Analyses of Different Preparations
centages of the Total Nitrogen.

From milk			Average				
Cow 2A1		Cow 10A1	From colostrum			From milk	
(9) Total globulin %	(10) Total globulin %	(11) Total globulin %	Eu-globulin (5 and 7) %	Pseudo-globulin (6 and 8) %	Total globulin (5, 6, 7 and 8) %	Total globulin (9, 10 and 11) %	Average of all seven samples %
8·03	8·04	8·22	7·10	7·26	7·18	8·10	7·57
2·23	1·95	2·30	2·20	2·12	2·16	2·16	2·16
25·80	26·82	25·99	24·74	24·24	24·49	26·20	25·23
2·01	2·00	1·59	2·02	1·82	1·92	1·87	1·90
10·59	11·30	10·92	10·81	10·56	10·68	10·94	10·79
3·92	4·13	4·68	3·78	3·70	3·74	4·24	3·96
9·28	9·39	8·80	8·13	8·16	8·15	9·16	8·58
62·89	62·54	63·55	64·64	65·20	64·92	63·00	64·10
61·39	61·52	62·52	63·77	63·90	63·83	61·81	62·97
1·50	1·02	1·03	0·87	1·30	1·09	1·19	1·13
98·95	99·35	100·06	98·68	98·81	98·74	99·46	99·06

TABLE III.

Summary of the Corrected Analyses of Different Preparations of Albumin in Percentages of Total Nitrogen.

	From colostrum			From milk		Average		Average of all five samples %
	Cow 2AI		Cow 10AI (14) %	Cow 2AI (15) %	Cow 10AI (16) %	Colostrum (12, 13 and 14) %	Milk (15 and 16) %	
	1st sample (12) %	2nd sample (13) %						
Ammonia-N	7·92	7·79	8·50	7·75	7·69	8·07	7·72	7·93
Melanin-N	1·97	1·88	1·95	1·50	1·80	1·93	1·65	1·82
Total N of bases ...	26·30	26·83	26·70	26·92	26·87	26·61	26·90	26·72
Cystine-N	1·90	2·32	2·00	2·49	2·19	2·07	2·34	2·18
Arginine-N	7·52	7·60	8·13	7·10	7·44	7·75	7·27	7·56
Histidine-N	4·41	4·61	4·78	4·20	4·21	4·60	4·20	4·44
Lysine-N	12·47	12·30	11·79	13·13	13·03	12·19	13·08	12·54
Total N of filtrate ...	63·06	61·40	62·12	62·89	62·97	62·19	62·93	62·49
Amino-N of filtrate ...	59·07	58·56	59·77	60·71	61·10	59·13	60·91	59·84
Non-amino-N of filtrate	3·99	2·84	2·35	2·18	1·87	3·06	2·03	2·65
Sum	99·25	97·90	99·27	99·06	99·33	98·80	99·20	98·96

Comparison of the Three Proteins.

In view of the divergent views which have been expressed from time to time as to the inter-relation of the proteins of milk it is of interest to compare the results of analysis, for which purpose Table IV gives a convenient summary.

TABLE IV.

	Caseinogen %	Lactoglobulin %	Lactalbumin %
Ammonia-N	10·25	7·57	7·93
Melanin-N	1·26	2·16	1·82
Total N of bases ...	26·62	25·23	26·72
Cystine-N	1·30	1·90	2·18
Arginine-N	9·31	10·79	7·56
Histidine-N	6·55	3·96	4·44
Lysine-N	9·46	8·58	12·54
Total N of filtrate ...	62·31	64·10	62·49
Amino-N of filtrate ...	55·44	62·97	59·84
Non-amino-N of filtrate	6·87	1·13	2·65

These results are conclusive as to the distinct chemical individuality of each of the three proteins. Thus caseinogen is sharply characterised by its relatively high proportion of ammonia-N, histidine-N and "non-amino-N of filtrate" (*i.e.* of proline-N + oxyproline-N + tryptophane-N) and relatively low proportion of melanin-N, cystine-N and α-mono-amino-acids ("amino-N of filtrate"). The arginine and lysine figures for caseinogen also mark it off as a separate protein.

Similarly the lactoglobulin is sharply differentiated from the lactalbumin by its lower content of lysine and its higher content of arginine. This large difference in lysine-content is in agreement with the observations of Hartley on the analogous proteins of ox-serum and horse-serum. The higher cystine-content of lactalbumin is in agreement with the sulphur-content of the two proteins. Thus, in lactalbumin from milk Sebelien found 1·73 % S, whilst in lactoglobulin from colostrum Tiemann [1898] found only 1·24 % S.

The foregoing conclusions as to the distinct chemical individuality of the three milk proteins are in complete accord with conclusions as to the biological differentiation of the three proteins which have been arrived at by the precipitin method [Hamburger, 1901], the method of complement deviation [Bauer and Engel, 1911] and anaphylactic methods [Besredka, 1909; Heuner, 1911].

TABLE V.

Showing the Similarity in Composition of the Globulins of Cow's Colostrum, Cow's Milk and Ox-Serum.

	Total globulin		Euglobulin		Pseudoglobulin		Average of all samples	
	From milk (average of three samples) (9, 10, 11) %	From ox-serum (one sample) (Hartley) %	From colostrum (average of two samples) (5, 7) %	From ox-serum (average of two samples) (Hartley) %	From colostrum (average of two samples) (6, 8) %	From ox-serum (one sample) (Hartley %	From milk and colostrum (seven samples) (5–11) %	From ox-serum (four samples) (Hartley) %
Ammonia-N	8·10	7·7	7·10	8·65	7·26	7·5	7·57	8·13
Melanin-N	2·16	2·0	2·20	2·25	2·12	1·9	2·16	2·10
Total N of bases ...	26·20	28·2	24·74	27·65	24·24	27·1	25·23	27·65
Cystine-N	1·87	2·0	2·02	1·7	1·82	1·9	1·90	1·82
Arginine-N	10·94	10·9	10·81	11·65	10·56	10·8	10·79	11·25
Histidine-N	4·24	6·3	3·78	5·15	3·70	4·8	3·96	5·35
Lysine-N	9·16	9·0	8·13	9·15	8·16	9·6	8·58	9·23
Total N of filtrate ...	63·00	62·0	64·64	60·05	65·20	63·3	64·10	61·35
Amino-N of filtrate ...	61·81	59·8	63·77	57·95	63·90	61·7	62·97	59·35
Non-amino-N of filtrate	1·19	2·2	0·87	2·1	1·30	1·6	1·13	2·00
Sum	99·46	99·9	98·68	98·60	98·81	99·8	99·06	99·23

Comparison with the Proteins of Ox-Serum.

The question of the relationship of the proteins of milk to those of the blood is one of obvious interest and has already received considerable attention. The earlier investigators, relying upon ultimate chemical analysis and methods of precipitation, concluded that the albumins of milk and of blood were identical. Later workers, using the biological methods elaborated by workers on immunity, have confirmed this conclusion and extended it further to the globulins [Bauer and Engel, 1911].

Our results obtained with lactoglobulin and lactalbumin, when taken in conjunction with those obtained by Hartley [1914] with ox-serum globulin and ox-serum albumin, whilst confirming the identity of the globulins, do not harmonise with the foregoing conclusions, so far as the albumins are concerned. For convenience of comparison we have assembled the data necessary for the comparison in Tables V and VI.

(*a*) *Comparison of Globulins.*

Table V contains a summary of the average results obtained by Hartley and by us in the analysis of the respective globulins.

Comparison of these results shows a striking resemblance throughout between the different preparations. The greatest variation is seen in the histidine values, but, as pointed out earlier, these are subject to relatively greater errors than any other of the values. If due allowance be made for unavoidable experimental error, and for the varying personal error of the different observers, there can be little doubt that these results, so far as they go, indicate that the respective globulins, whether total globulin, pseudo-globulin, or euglobulin, of colostrum and milk are identical with those of ox-serum.

(*b*) *Comparison of Albumins.*

The average results obtained by us for lactalbumin are summarised in Table VI, together with Hartley's analysis of serum albumin. This table also includes in the last column the average of three analyses by van Slyke [1915] of a sample of lactalbumin obtained from milk by precipitation of the caseinogen by acetic acid and coagulation of the lactalbumin by heating. This preparation would obviously be contaminated with the small amount of lactoglobulin present in the milk, but the analysis serves as a useful check upon our results obtained with purer preparations.

TABLE VI.

Showing the Differences in Composition of the Albumins of Cow's Milk and Ox-Serum.

	Lactalbumin			Serum-albumin	Lactalbumin
	From colostrum (average of three samples, 12–14) %	From milk (average of two samples, 15, 16) %	Average of all five samples (12–16) %	One sample (Hartley) %	Average of three samples (van Slyke) %
Ammonia-N	8·07	7·72	7·93	5·8	8·57
Melanin-N	1·93	1·65	1·82	1·1	2·32
Total N of bases ...	26·61	26·90	26·72	36·9	25·31
Cystine-N	2·07	2·34	2·18	3·5	1·30
Arginine-N	7·75	7·27	7·56	10·4	7·20
Histidine-N	4·60	4·20	4·44	6·7	4·57
Lysine-N	12·19	13·08	12·54	16·3	12·24
Total N of filtrate ...	62·19	62·93	62·49	56·5	63·50
Amino-N of filtrate ...	59·13	60·91	59·84	54·2	61·53
Non-amino-N of filtrate	3·06	2·03	2·65	2·3	1·97
Sum	98·80	99·20	98·96	100·3	99·70

Comparison of the results reveals marked differences between our lactalbumin and Hartley's serum albumin. This is strikingly manifest in every item of the analyses, but notably in the basic nitrogenous constituents. The reliability of our results for lactalbumin may be gauged from the separate analyses set out in Table III, and further from the reasonably close concordance with van Slyke's results. On the other hand, we have for serum albumin only the one analysis by Hartley, although his results are substantially confirmed by those obtained by him with albumin from horse-serum.

As already mentioned most of the older chemical work and the more recent biological work has pointed to the identity of lactalbumin with serum albumin, but observations that do not harmonise with this conclusion have not been lacking.

Thus Sebelien [1885] found that albumin whether prepared from colostrum or milk had a specific rotatory power of $[\alpha]_D = -36°$ to $-37°$, whilst serum albumin isolated by a similar method from ox-blood gave $[\alpha]_D = -62{\cdot}6°$. Further, Halliburton [1890] showed that lactalbumin differed from serum albumin in its behaviour on heat-coagulation, and in its precipitability by certain neutral salts.

There is obvious need for further investigation here, but it is clear that biological tests alone will not suffice for the purpose. With the material at our disposal we propose to investigate this point more closely.

Experimental Details.

Preparations of the Proteins.

Summary. *Caseinogen* was prepared by precipitation from the diluted colostrum or milk with saturated potash alum solution (Schlossmann); *Total Globulin* by saturation of the neutralised filtrate from the caseinogen with anhydrous magnesium sulphate; the two globulins were separated by dialysis; *Albumin* was obtained from the filtrate from the total globulin by saturation with anhydrous sodium sulphate at 40° C. (Pinkus). Ammonium sulphate was not used as a precipitant for the globulin and albumin in order to avoid the possibility of adsorption of extraneous nitrogen by the proteins.

Caseinogen. Freshly drawn colostrum was diluted with five times its volume of *distilled* water[1] and heated to 40° in order to produce a more satisfactory settling of the caseinogen. Saturated potash alum solution was then added in sufficient amount to produce complete precipitation of the caseinogen. In the case of milk, dilution with only three times its volume of water was necessary and less alum solution was also needed. After settling for half an hour, the clear supernatant liquid was syphoned off, the residuum of caseinogen and fat strained on muslin, washed three times by stirring vigorously with water, and the filtered washings added to the main bulk of liquid, for the preparation of globulin and albumin.

Before proceeding with the purification of the caseinogen, it was necessary to free it from the large amount of fat carried down with it on precipitation. This was done at first by extracting the semi-solid mass with alcohol and then with ether. It was found preferable, however, to extract the fat with ether from the solution of caseinogen in alkali. For this purpose the caseinogen, suspended in distilled water, which was kept well stirred, was dissolved in the minimum amount of 1% soda and the solution extracted with ether. The ethereal layer carried with it most of the insoluble matter, and filtration of the solution of caseinogen in alkali was thereby rendered much easier. The filtered solution was diluted with distilled water and the caseinogen reprecipitated with 1% acetic acid, added with constant stirring. After settling, the supernatant liquid was syphoned off and the caseinogen washed by sedimentation. This process of dissolving in alkali and reprecipitating was repeated five times. The final solution in alkali was filtered clear through

[1] It is essential, in order to produce a perfectly white sample of caseinogen, that all dilutions should be done with distilled water. The use of tap water, especially if of peaty origin, causes the production of a brownish coloured product.

filter paper pulp, diluted, and precipitated with the minimum amount of 1% hydrochloric acid. After washing and draining, the caseinogen was dried by grinding with alcohol, and finally with ether. The final product (air-dried) was a perfectly white, dusty powder.

Globulin. The filtrate from the caseinogen preparation was neutralised with dilute soda, and saturated with anhydrous magnesium sulphate, thus obviating the large increase in volume which occurs if the crystalline salt is used. The precipitated globulin was filtered off on large Buchner funnels and the filtrate used for the isolation of albumin. The crude globulin was dissolved in distilled water to which a little magnesium sulphate had been added, the solution filtered through pulp, saturated with magnesium sulphate, and the globulin filtered off. This reprecipitation was repeated four times at the same volume each time, and the filtrate in each case was added to the albumin preparation. The final solution of globulin was then dialysed in large sausage-skin dialysers for six days in running water, and then in relays of distilled water until free from sulphates. The globulin was thus separated into two fractions; euglobulin, insoluble in distilled water, and pseudoglobulin, soluble in distilled water.

Euglobulin. This was filtered from the soluble pseudoglobulin, dissolved in the minimum amount of 0·6% sodium chloride solution, filtered, and reprecipitated by diluting the solution with ten times its volume of distilled water. The precipitate was allowed to settle, washed with distilled water, and finally dried with alcohol and ether, thus giving an almost white powder.

Pseudoglobulin. The filtered solution of pseudoglobulin in water was precipitated with alcohol, filtered, and the protein dried with alcohol and ether, giving a perfectly white powder.

Albumin. This protein was isolated and purified by Pinkus' method [1901]. The combined filtrates from the globulin preparation were heated to 40°, saturated with anhydrous sodium sulphate (free from chlorides) and allowed to stand at this temperature for a few hours. The precipitated albumin was then filtered off through a warm jacketed filter, care being taken to prevent the salt from crystallising. The crude albumin was dissolved in cold water, filtered, and the solution saturated with magnesium sulphate. The slight precipitate, probably globulin, was filtered off, and the filtrate saturated with anhydrous sodium sulphate at 40° as before. This process was repeated four times, saturation with magnesium sulphate being from the same volume each time. The last saturation with magnesium sulphate gave no precipitate. The final precipitate of lactalbumin was dissolved in water

and the filtered solution precipitated with alcohol, the protein being finally dried with alcohol and ether, and gave a white, light powder, readily soluble in water.

In the preparation of the proteins from colostrum the first three milkings after calving from two cows (2AI and 10AI) were taken, and the proteins isolated from each of these separately. Had any appreciable differences between the respective proteins of colostrum and milk been found, it was proposed to analyse separately each sample of protein obtained. Since, however, no such differences could be detected, only the preparations from the first sample of colostrum after calving were analysed.

The proteins from milk were prepared from samples taken from the same cows three months after calving, when it could be safely assumed that the milk had become quite normal. The method of isolation was the same as for colostrum. It has been remarked, however, that in the purification of the albumin from colostrum, when the solution of the albumin was saturated with magnesium sulphate, it gave in the first two or three reprecipitations a slight precipitate with magnesium sulphate, which was supposed to be a globulin. In working up the colostrum these small amounts of globulin were rejected, an ample yield being otherwise obtained. In the case of milk, however, the combined precipitates obtained by saturation with magnesium sulphate in the purification of the albumin, were added to the main bulk of the globulin precipitate, and the whole purified by reprecipitation with magnesium sulphate six times. That it was really a part of the globulin which remained dissolved in the albumin solution is clear from the analyses, and the recovery of these portions probably accounts for the fact that a much bigger yield of globulin was obtained from milk than has hitherto been described. Twenty litres of milk from each cow were worked up separately, but only a portion of the caseinogen was purified. The following amounts of lactoglobulin and lactalbumin were isolated. From 20 litres of milk in each case

Cow 2AI gave 7·28 g. lactoglobulin and 43·0 g. lactalbumin.

Cow 10AI gave 4·54 g. lactoglobulin and 35·4 g. lactalbumin.

Owing to the difficulty of obtaining more than small quantities of the globulin in milk, and since preliminary analyses had pointed to the fact that euglobulin and pseudoglobulin from colostrum are indistinguishable by the method of analysis used, the samples of globulin obtained from milk were not fractionated into eu- and pseudoglobulin, so that in this case data for total globulin only are recorded in the tables.

Methods of Analysis.

Van Slyke's method of protein analysis was used throughout these experiments. For details the original paper must be consulted.

In order that the analyses might be strictly comparable the conditions of analysis of each protein were made as nearly the same as possible. Whenever the amount available permitted it, 3·5 g. of the protein, air dried, were used for an analysis, the lowest amount used (analysis 10, Table VII) being 2·5 g. In all cases the protein was hydrolysed by heating with thirty-five times its weight of 20 % hydrochloric acid for 48 hours at 100°, using flasks with air-condensers ground into the necks. The resultant mixture was evaporated *in vacuo* to remove as much HCl as possible and was then made up to 100 cc., and the following estimations were made:

(i) *Total N* estimated (Kjeldahl) on two separate portions of 10 cc. each.

(ii) 75 cc. were taken for the subsequent analysis and gave

(*a*) *Ammonia-N* in the 75 cc.

(*b*) *Melanin-N* in the 75 cc.

After removal of the ammonia and melanin the bases were precipitated with phosphotungstic acid, and after removal of the phosphotungstic acid with baryta, the solution of the bases was made up to 100 cc., and the following estimations made on this solution:

(*c*) *Cystine-N* in 20 cc. of the solution of the bases.

(*d*) *Arginine-N* in 50 cc. of the solution of the bases.

(*e*) *Residual-N* (*i.e.* half the arginine-N and total N of cystine, histidine, and lysine) in the same 50 cc. of solution as (*d*), so that (*d*) + (*e*) gave the total N of the bases.

(*f*) *Amino-N of the bases* in 2 cc. of the solution: done in triplicate.

The filtrate from the phosphotungstic acid precipitate of the bases was made up to 200 cc. and the following estimations were made on this solution:

(*g*) *Total N of the filtrate* in 50 cc.: done in duplicate.

(*h*) *Amino-N of the filtrate*; 15 cc. were diluted to 25 cc. and the amino-N estimated in 2 cc.: done in triplicate.

(*i*) *Non-amino-N of the filtrate*, was calculated by taking the differences of (*g*) and (*h*).

Table VII contains a record of the whole of the analytical observations performed on each protein, as described in the above scheme, whilst Table VIII gives a summary of the whole of the results obtained.

Nature of preparation analysed	Total N		Ammonia		Melanin		Cystine	
	cc. thio. per 10 cc.	g. N per 75 cc.	cc. thio. per 75 cc.	g. N per 75 cc.	cc. thio. per 75 cc	g. N per 75 cc.	$BaSO_4$ g. per 20 cc.	g. N per 75 cc.
1. Caseinogen from colostrum. Cow 2AI ...	(34·63) (34·48)	0·3545	27·02	0·03695	3·35	0·004581	0·0071	0·00213
2. Caseinogen from colostrum. Cow 10AI ...	(33·61) (33·84)	0·3458	25·45	0·03480	3·41	0·004663	0·0075	0·00225
3. Caseinogen from milk. Cow 2AI	(36·61) (36·69)	0·3777	27·89	0·03832	3·51	0·004822	0·0068	0·00204
4. Caseinogen from milk. Cow 10AI ...	(34·88) (34·85)	0·3577	27·07	0·03701	2·92	0·003993	0·0061	0·00183
5. Euglobulin from colostrum. Cow 2AI ...	(37·39) (37·27)	0·3922	20·39	0·02856	5·61	0·007859	0·0175	0·00525
6. Pseudoglobulin from colostrum. Cow 2AI	(37·43) (37·53)	0·3939	20·20	0·02830	5·70	0·007986	0·0154	0·00462
7. Euglobulin from colostrum. Cow 10AI ...	(38·25) (38·25)	0·4020	19·84	0·02780	6·89	0·009651	0·0186	0·00558
8. Pseudoglobulin from colostrum. Cow 10AI	(38·50) (38·71)	0·4056	21·23	0·02974	6·37	0·008923	0·0157	0·00471
9. Total globulin from milk. Cow 2AI ...	(36·46) (36·43)	0·3807	22·45	0·03058	6·08	0·008470	0·0168	0·00504
10. Total globulin from milk. Cow 2AI ...	(25·35) (25·59)	0·2661	15·33	0·02139	3·72	0·005182	0·0091	0·00273
11. Total globulin from milk. Cow 10AI ...	(30·72) (30·72)	0·3210	18·91	0·02638	5·29	0·007370	0·0083	0·00249
12. Albumin from colostrum. Cow 2AI ...	(28·79) (28·69)	0·3003	17·46	0·02378	4·24	0·005907	0·0103	0·00309
13. Albumin from colostrum. Cow 2AI ... (Duplicate of 12.)	(28·61) (28·59)	0·2988	16·68	0·02328	4·04	0·005628	0·0144	0·00432
14. Albumin from colostrum. Cow 10AI ...	(33·76) (33·76)	0·3528	22·01	0·02997	4·95	0·006900	0·0148	0·00444
15. Albumin from milk. Cow 2AI	(34·97) (34·93)	0·3584	20·31	0·02777	3·90	0·005333	0·0211	0·00633
16. Albumin from milk. Cow 10AI	(34·88) (34·84)	0·3538	19·98	0·02722	4·57	0·006366	0·0171	0·00513

TABLE VII. *Details of Analyses.*

ine	Arginine		Total N of bases			Amino-N of bases				Amino-N of filtrate			
g. N per 75 cc.	cc. thio. per 50 cc.	g. N per 75 cc.	cc. thio. per 50 cc.	cc. thio. per 50 cc. + arginine titration	g. N per 75 cc.	cc. gas per 2 cc.	$t°$	p mm.	g. N per 75 cc.	cc. gas per 2 cc.	$t°$	p mm.	g. N p 75 c
0·00213	5·12	0·02853	24·26	29·38	0·08186	1·952 1·948 1·940	17·0 16·4 16·0	770·3 770·6 770·7	0·04774	2·375 2·363 2·388	19·0 18·2 18·8	765·7 765·1 764·4	0·2069
0·00225	5·55	0·03093	24·04	29·59	0·08244	1·965 1·955 1·940	16·1 15·6 17·0	763·5 764·0 770·6	0·04766	2·265 2·255 2·252	18·6 18·1 18·0	767·3 767·3 767·3	0·1966
0·00204	5·97	0·03356	27·41	33·38	0·09380	2·158 2·160 2·170	15·7 16·0 16·2	757·6 757·6 757·0	0·05312	2·347 2·380 2·355	13·1 14·2 14·1	761·0 761·0 761·0	0·2094
0·00183	5·02	0·02796	25·10	30·12	0·08391	2·020 2·028 2·023	12·7 13·0 15·7	761·4 761·4 763·4	0·05004	2·332 2·336 2·335	18·8 19·2 19·0	768·2 768·0 768·0	0·2038
0·00525	7·06	0·03968	24·17	31·23	0·08776	2·085 2·070 2·075	15·4 15·3 14·6	749·5 750·0 755·0	0·05041	2·900 2·905 2·880	15·0 15·0 13·5	753·3 753·2 755·0	0·2586
0·00462	6·93	0·03895	23·91	30·84	0·08667	2·015 2·020 2·045	14·7 15·0 14·9	754·8 754·8 746·6	0·04907	2·910 2·905 2·915	14·7 15·0 14·6	752·6 754·7 754·7	0·2592
0·00558	7·08	0·03979	24·39	31·47	0·08844	2·135 2·135 2·115	15·5 15·9 16·0	746·3 746·3 747·0	0·05129	2·900 2·902 2·880	15·0 15·0 15·0	756·2 756·2 761·2	0·2588
0·00471	6·94	0·03900	23·94	30·88	0·08677	2·125 2·110 2·100	15·0 16·0 16·1	750·6 751·8 752·0	0·05127	2·900 2·900 2·890	15·0 13·2 13·0	760·6 762·0 762·0	0·2620
0·00504	6·66	0·03712	24·96	31·62	0·08811	2·150 2·140 2·140	18·2 17·8 17·6	767·2 767·2 767·2	0·05286	2·713 2·697 2·718	15·0 14·5 14·8	751·7 751·4 750·8	0·2389
0·00273	4·82	0·02686	17·18	22·00	0·06130	1·575 1·585 1·580	18·0 18·1 17·9	765·8 765·3 765·7	0·03637	1·965 1·975 1·965	13·8 14·0 13·0	750·0 750·0 755·8	0·1689
0·00249	5·72	0·03187	20·60	26·32	0·07333	1·770 1·775 1·778	18·1 17·9 17·7	764·3 764·7 765·0	0·04196	2·355 2·355 2·360	15·4 14·1 14·7	754·4 754·4 754·0	0·2059
0·00309	3·48	0·01939	21·23	24·71	0·06886	1·967 1·955 1·970	16·9 17·0 16·0	768·8 768·8 768·2	0·04802	2·142 2·128 2·126	19·2 19·0 19·0	763·4 763·1 763·1	0·1826
0·00432	3·50	0·01950	21·64	25·14	0·07005	2·004 1·997 1·985	18·0 17·6 16·7	768·8 768·8 770·0	0·04877	2·095 2·105 2·105	17·6 17·9 17·9	760·7 760·2 760·2	0·1802
0·00444	4·57	0·02547	25·59	30·16	0·08405	2·245 2·258 2·265	16·7 18·2 17·6	768·6 767·8 766·3	0·05624	2·478 2·476 2·482	17·5 16·9 17·4	758·0 757·8 757·3	0·2161
0·00633	3·99	0·02224	27·02	31·01	0·08640	2·480 2·505 2·490	17·7 17·0 17·8	758·4 757·6 759·4	0·06222	2·565 2·578 2·568	16·8 16·7 17·5	751·2 751·2 751·2	0·2228
0·00513	4·15	0·02313	26·35	30·50	0·08498	2·432 2·435 2·420	18·9 18·7 17·1	758·8 758·8 759·3	0·06022	2·540 2·535 2·530	16·0 15·8 15·6	751·5 752·6 753·2	0·2214

	Total N of filtrate		Total N recovered
g. N per 75 cc.	cc. thio. per 50 cc.	g. N per 75 cc.	g. per 75 cc.
0·2069	{42·04 / 42·11}	0·2345	0·3579
0·1966	{40·50 / 40·40}	0·2254	0·3473
0·2094	{43·09 / 43·06}	0·2405	0·3774
0·2035	{42·03 / 42·04}	0·2343	0·3592
0·2580	{47·41 / 47·39}	0·2663	0·3905
0·2592	{47·96 / 47·91}	0·2693	0·3923
0·2588	{47·58 / 47·58}	0·2673	0·3932
0·2620	{48·45 / 48·40}	0·2720	0·3974
0·2389	{44·69 / 44·83}	0·2495	0·3767
0·1689	{31·67 / 31·66}	0·1765	0·2644
0·2059	{38·42 / 38·44}	0·2141	0·3212
0·1826	{35·75 / 35·83}	0·1995	0·2980
0·1802	{34·72 / 34·72}	0·1936	0·2926
0·2161	{41·17 / 41·11}	0·2293	0·3502
0·2228	{42·26 / 42·26}	0·2355	0·3550
0·2214	{41·82 / 41·79}	0·2329	0·3515

TABLE VIII. *Results of Analyses expressed in Percentages of the Total Nitrogen.*

(*a*) *Uncorrected.* (*b*) *Corrected for solubility of the Phosphotungstates of Bases.*

Protein analysed		Ammonia N	Melanin N	Total N of bases	Cystine N	Arginine N	Histidine N	Lysine N	Total N of filtrate	Amino N of filtrate	Non-amino N of filtrate	Sum
1. Caseinogen (colostrum). Cow 2AI	(*a*)	10·42	1·29	23·09	0·60	8·05	5·38	9·06	66·15	58·38	7·77	100·95
	(*b*)	10·42	1·29	25·94	1·34	8·95	6·45	9·20	63·30	56·90	6·40	
2. Caseinogen (colostrum). Cow 10AI	(*a*)	10·07	1·35	23·83	0·65	8·94	5·02	9·22	65·18	56·86	8·32	100·43
	(*b*)	10·07	1·35	26·76	1·40	9·87	6·12	9·37	62·25	55·34	6·91	
3. Caseinogen (milk). Cow 2AI	(*a*)	10·15	1·27	24·84	0·54	8·89	6·16	9·25	63·68	55·46	8·22	99·94
	(*b*)	10·15	1·27	27·52	1·23	9·73	7·17	9·39	61·00	54·06	6·94	
4. Caseinogen (milk). Cow 10AI	(*a*)	10·35	1·12	23·46	0·51	7·82	5·41	9·72	65·52	56·93	8·59	100·45
	(*b*)	10·35	1·12	26·28	1·24	8·71	6·47	9·86	62·70	55·46	7·24	
5. Euglobulin (colostrum). Cow 2AI	(*a*)	7·28	2·00	22·39	1·34	10·12	2·91	8·02	67·89	65·79	2·10	99·54
	(*b*)	7·28	2·00	24·95	2·00	10·93	3·87	8·15	65·31	64·46	0·85	
6. Pseudoglobulin (colostrum). Cow 2AI	(*a*)	7·19	2·03	22·01	1·17	9·89	3·20	7·75	68·36	65·83	2·53	99·61
	(*b*)	7·19	2·03	24·57	1·83	10·70	4·16	7·88	65·82	64·49	1·33	
7. Euglobulin (colostrum). Cow 10AI	(*a*)	6·92	2·40	22·01	1·39	9·90	2·73	7·99	66·48	64·39	2·09	97·81
	(*b*)	6·92	2·40	24·52	2·04	10·69	3·68	8·11	63·97	63·08	0·89	
8. Pseudoglobulin (colostrum). Cow 10AI	(*a*)	7·33	2·20	21·40	1·16	9·62	2·31	8·31	67·07	64·60	2·47	98·00
	(*b*)	7·33	2·20	23·90	1·81	10·41	3·25	8·43	64·57	63·31	1·26	
9. Total globulin (milk). Cow 2AI	(*a*)	8·03	2·23	23·15	1·32	9·75	2·92	9·16	65·52	62·75	2·77	98·95
	(*b*)	8·03	2·23	25·80	2·01	10·59	3·92	9·28	62·89	61·39	1·50	
10. Total globulin (milk). Cow 2AI	(*a*)	8·04	1·95	23·03	1·02	10·09	2·70	9·22	66·33	63·48	2·85	99·35
	(*b*)	8·04	1·95	26·82	2·00	11·30	4·13	9·39	62·54	61·52	1·02	
11. Total globulin (milk). Cow 10AI	(*a*)	8·22	2·30	22·85	0·78	9·93	3·49	8·65	66·71	64·15	2·56	100·06
	(*b*)	8·22	2·30	25·99	1·59	10·92	4·68	8·80	63·55	62·52	1·03	
12. Albumin (colostrum). Cow 2AI	(*a*)	7·92	1·97	22·93	1·03	6·46	3·14	12·30	66·40	60·78	5·62	99·25
	(*b*)	7·92	1·97	26·30	1·90	7·52	4·41	12·47	63·06	59·07	3·99	
13. Albumin (colostrum). Cow 2AI	(*a*)	7·79	1·88	23·46	1·45	6·53	3·34	12·14	64·77	60·30	4·47	97·90
	(*b*)	7·79	1·88	26·83	2·32	7·60	4·61	12·30	61·40	58·56	2·84	
14. Albumin (colostrum). Cow 10AI	(*a*)	8·50	1·95	23·83	1·26	7·22	3·70	11·65	65·00	61·25	3·75	99·27
	(*b*)	8·50	1·95	26·70	2·00	8·13	4·78	11·79	62·12	59·77	2·35	
15. Albumin (milk). Cow 2AI	(*a*)	7·75	1·50	24·11	1·77	6·21	3·14	12·99	65·70	62·18	3·52	99·06
	(*b*)	7·75	1·50	26·92	2·49	7·10	4·20	13·13	62·89	60·71	2·18	
16. Albumin (milk). Cow 10AI	(*a*)	7·69	1·80	24·02	1·45	6·54	3·14	12·89	65·83	62·56	3·27	99·33
	(*b*)	7·69	1·80	26·87	2·19	7·44	4·21	13·03	62·97	61·10	1·87	

Observations on the Method of Analysis.

(i) In all estimations of nitrogen by the Kjeldahl method the excess of acid used for neutralising the ammonia was determined by adding to the liquid to be titrated a little solid potassium iodide and then a few cc. of a saturated solution of normal potassium iodate. The iodine liberated by the free acid present was then estimated by titration with standard (N/10) sodium thiosulphate, using starch paste as indicator. A sharper end-point was thereby obtained than with the ordinary acidimetric method of titration. The strength of the thiosulphate solution was determined at short intervals by checking it against the standard acid used, and by titration with weighed amounts of pure iodine.

(ii) The filtration of the gelatinous melanin precipitate produced by the lime is very slow if carried out on an ordinary filter paper. It was found to be performed more expeditiously and the precipitate needed less washing to free it from chlorides by filtering by suction. In this, as in the filtration and washing of the phosphotungstates of the bases, the filtering tube as described by Irvine [1915] was used. A double gravimetric filter paper was fitted into the Buchner funnel, and a very slightly reduced pressure maintained all the time in the tube. The precipitate, if not allowed to become dry, when it cracks, is thus easily and quickly washed free from chlorides.

(iii) In the estimation of amino-nitrogen, van Slyke's micro-apparatus [1914] was used throughout. By this means all the estimations could be done in triplicate, and thus with greater accuracy than with the large apparatus. Increased accuracy was further obtained by enclosing the gas measuring burette in a glass jacket through which cold water of known temperature was slowly circulated, so that the volume of gas could be conveniently read off without any disturbance of its temperature. By this means, and with the aid of a lens in taking readings—the burette being of 5 cc. capacity with divisions of 0·01 cc.—it was found possible to read the volume of the gas to 0·002 cc. and to obtain triplicates agreeing within 0·01 cc. The liquid to be analysed was measured from a calibrated 2 cc. pipette and washed into the apparatus with a few drops of distilled water.

(iv) *Corrections for Reagents.* The following were the corrections determined for the impurities in the reagents. All the readings in Table VII have been corrected except the amino-nitrogen estimations.

Total N. 0·16 cc. thio. *Ammonia-N* nil. *Melanin-N.* 0·24 cc. thio.
Cystine-N. 0·0023 g. $BaSO_4$. *Arginine-N.* 0·16 cc. thio.

Residual N of bases 0·18 cc. thio. *Amino-N of bases* 0·188 mgm. N.

Total N of filtrate 0·18 cc. thio. *Amino-N of filtrate* 0·129 mgm. N.

(v) *Corrections for the solubility of the phosphotungstates of the bases.* The phosphotungstates of the bases are slightly soluble in the liquid from which they are precipitated. In order to correct for this error the bases were always precipitated from the same volume of solution (200 cc.) and the corrections as determined by van Slyke [1911, p. 32] applied.

The analyses given in Tables I–VI have all been corrected in this way, while Table VIII contains a summary of the analyses, expressed as percentages of the total nitrogen, before and after correction.

Summary.

The chief proteins of cow's colostrum and cow's milk were isolated, and analysed by the method of van Slyke, with the following results.

1. Caseinogen, total lactoglobulin, and lactalbumin are sharply differentiated, distinct proteins, and have respectively the same composition whether prepared from colostrum or normal milk.

2. The presence in milk of a globulin in small quantities (0·03 % isolated) was proved.

3. Eu-lactoglobulin and pseudo-lactoglobulin are identical so far as the protein part of their molecules is concerned.

4. Lactoglobulin from either colostrum or milk is very closely allied to, and is probably identical with, serum globulin from ox-blood.

5. Lactalbumin from either colostrum or milk is very different in composition from serum albumin from ox-blood.

A considerable portion of the work described in this communication has been carried out in the Animal Nutrition Research Laboratory of the School of Agriculture, Cambridge, and we would take this opportunity of acknowledging our indebtedness to Professors Wood and Hopkins for the facilities kindly accorded for the purpose.

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XXXVI. THE ACTION OF HYPOCHLORITES AND ALLIED SUBSTANCES ON PROTEINS, AND THEIR BEHAVIOUR ON INJECTION.

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(*Received August 17th, 1916.*)

The recent work of Lorrain Smith [1915, 1, 2], Dakin [1915, 1, 2, 1916] and others has directed attention to the value of substances containing chlorine in "available" form as germicidal agents in the treatment of infected wounds. Although such substances have long been recognised as powerfully germicidal, they were not previously made use of to any large extent owing to the decrease in their efficiency in the presence of albuminous material. When small quantities of such a fluid as blood serum are added to hypochlorite solutions there is, even at comparatively low temperatures, an initial rapid lowering in the "available" chlorine value followed by a much slower rate of loss. The initial rapid loss is evidently due to oxidation changes and to the passage of chlorine into the cyclic rings of such protein derivatives as tyrosine etc., the hydrogen attached to the carbon being replaced. Part of the chlorine however, especially at low temperatures, replaces the hydrogen attached to nitrogen in various primary and secondary amine groups, forming compounds containing . N : Cl_2 or : N . Cl groups. As the chlorine in these groups is still in active form, such compounds, if formed under the ordinary conditions of wound treatment or after intravascular injection, would probably constitute valuable germicidal agents. The significance and value of these chloramino-derivatives of protein must depend in the first place on the rate of formation and concentration of these bodies existing in the wound or in the blood, and, in the second place, on the rate at which these compounds lose their available chlorine either by hydrolysis or by transference to the cyclic rings.

In order to gain some knowledge of the changes which hypochlorites and

aromatic substances containing chloramine groups undergo in the presence of proteins, it is necessary to carry out the tests at the temperature to which these bodies would be exposed in wound treatment. A series of experiments was therefore carried out to determine the percentage loss of available chlorine, under the above mentioned conditions, at stated intervals during the reaction. From such a study information may also be obtained as to the order of the chemical reactions concerned, but in this communication very little reference will be made to this part of the subject.

The Action of Hypochlorites on Albuminous Solutions.

Even extremely weak solutions of free HOCl precipitate albumins but solutions of the hypochlorites in general use have not this effect. At body temperature there is however a rapid disintegration of the protein, nitrogen coming freely off and the amino-acids breaking down into aldehydes or ketones with one carbon atom less than the original acids. The odour of aldehyde formation is very characteristic of the action of hypochlorites on proteins. The rate of change of all hypochlorite solutions in the presence of albumin is approximately the same, and as I was engaged in the study of the action of magnesium hypochlorite as a possibly less irritant antiseptic than sodium or calcium hypochlorite, the majority of the experiments were carried out with this solution.

The method of preparation of $Mg(OCl)_2$ was the following: hypochlorous acid was prepared from bleaching powder and boric acid used in the following proportions, 10 g. bleaching powder, 300 cc. water, and 15–20 g. boric acid. Distillation took place in vacuo and the distillate was collected in a vessel containing an excess of freshly precipitated and washed $Mg(OH)_2$, the receiver being kept in an ice-salt mixture. From 50–100 cc. were distilled over, and the solution so obtained contained 4–5 g. HOCl per litre. These solutions are quite stable and may be kept for at least a month. A similar solution may be conveniently and rapidly prepared by the addition of magnesium sulphate solution to one of bleaching powder, the precipitate being allowed to settle and the fluid siphoned off. Such a solution has been used with advantage as a fairly non-irritant antiseptic by Charles Mayer [1916].

The rate of change of hypochlorite in the presence of albumin was determined in the following way. The hypochlorite solution in a stoppered bottle was brought up to a temperature of 36° in a thermostat which also

contained a vessel with blood serum. The "available" chlorine value of the former was estimated and when both solutions had reached the proper temperature, a small fixed quantity of the serum was added to a known volume of the warmed hypochlorite. At definite intervals the available chlorine value of the mixture was determined by running a measured quantity of the mixture into potassium iodide solution acidified with acetic acid and titrating the iodine set free with N/10 thiosulphate. In order to determine the type of the reaction estimations of the chlorine loss were carried out with different concentrations of hypochlorite the quantity of serum remaining constant, and also with hypochlorite solutions of the same strength but with varying quantities of serum.

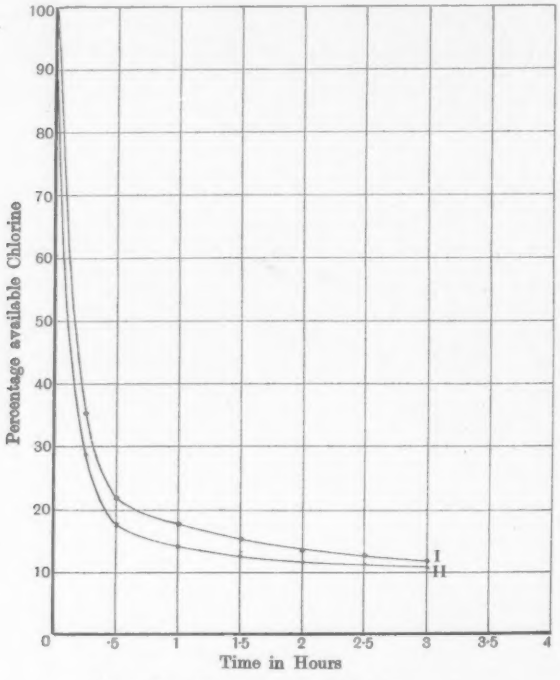
Before referring to the curves of fall in concentration of active chlorine in the presence of serum, it is necessary to gain some idea of the amount of hypochlorite which can be used up at room temperature by a given amount of protein when an excess of the former is used and when sufficient time is given to complete the reaction. Effront [1905] has calculated that 1 mgm. protein (egg white) uses up 3·055 mgm. active chlorine, or for 1 mgm. nitrogen of the protein 18·629 mgm. chlorine. Using the same method as Effront, I have found that 1 mgm. nitrogen of blood serum uses up 15·87 mgm. $Mg(OCl)_2$. Therefore when 5 cc. serum are taken containing 61 mgm. nitrogen about 0·968 g. $Mg(OCl)_2$ can be used up. 200 cc. $Mg(OCl)_2$ $m/20$ contain 1·2722 g. $Mg(OCl)_2$, so that if 5 cc. serum were added to this solution there would be at the end of the reaction a slight excess of hypochlorite, while with the weaker hypochlorite solutions or with higher protein concentrations, the protein would be in excess[1].

Fig. 1 shows the available chlorine fall when 200 cc. of $m/20$ $Mg(OCl)_2$ (Graph I) and 200 cc. $m/40$ $Mg(OCl)_2$ (Graph II) respectively are mixed with 5 cc. blood serum (containing 0·061 g. nitrogen) the mixture being kept at 36°. Control solutions of $Mg(OCl)_2$ of the same strength without the addition of serum were also kept under the same temperature conditions and the available chlorine determined at the stated intervals. The control solutions showed practically no alteration during the period of the experiment.

The curves show a fall down to 30–35 % of the original value within 15 minutes, but after the first half hour the rate of loss of available chlorine

[1] The interesting observations of Dakin [1916] published after the completion of my work show that with higher protein concentrations than I used with hypochlorite solutions, namely from 1 cc. up to 10 cc. serum added to 5 cc. neutral hypochlorite containing 0·0126 g. HOCl, more active chlorine disappears in a given time with the smaller amounts of serum than with the larger.

becomes very much slower. The curves are of the same type, slightly the greater fall taking place with the weaker solution. At the end of three hours, the available chlorine value is only slightly over 10 % of the initial concentration and is approximately the same in both cases. The curves show falls which are practically independent of the initial concentration.

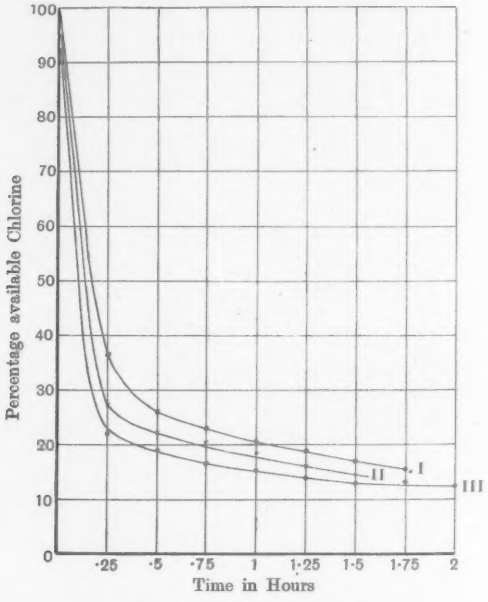


200 cc. Mg $(OCl)_2$ $m/20$ + 5 cc. Blood Serum = I
" " $m/40$ + " " " = II

Fig. 1.

Fig. 2 shows the effect of varying the protein and keeping the initial hypochlorite concentration the same. Three 200 cc. portions of $m/20$ Mg $(OCl)_2$ were taken and 5 cc. (Graph I) 10 cc. (Graph II) and 20 cc. serum (Graph III) respectively were added to the solutions. The available chlorine value in the first case fell to 37 % of the initial value, in the second to 28 % and in the third to 22 % within 15 minutes of admixture. This primary rapid fall is succeeded by the characteristic slow fall observed in Fig. 1. The types of curve are the same and at the end of 2 hours the active chlorine

is about 12–15 % of the initial value in the three solutions. The fall continues slowly during the next 24 hours.



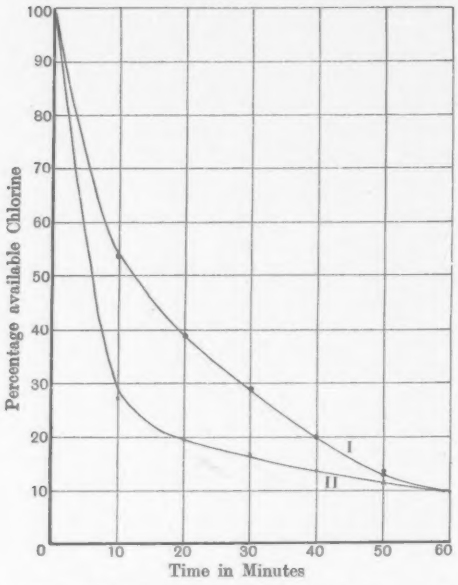
200 cc. $Mg(OCl)_2$ *m*/20+ 5 cc. Serum = I
" " " +10 cc. " = II
" " " +20 cc. " = III

Fig. 2.

In Fig. 3 the fall is plotted out for shorter intervals and for solutions of eusol (Graph I) of a strength which is commonly used as an antiseptic (0·315 % "available" chlorine) and $Mg(OCl)_2$ (0·556 % "available chlorine") (Graph II).

In the case of the former 5 cc. serum were added and in the latter 9 cc.

In the case of Graph I, Fig. 3 apart from the fall during the first 10 minutes, the logarithm of the concentration is practically proportional to the time—a reaction of the first order. At the close of the first hour the chlorine value in both cases is only 10 % of the initial figure. From this point onwards the fall becomes very slow in both cases so that 18 hours later the chlorine value is still 3 % of the initial figure.



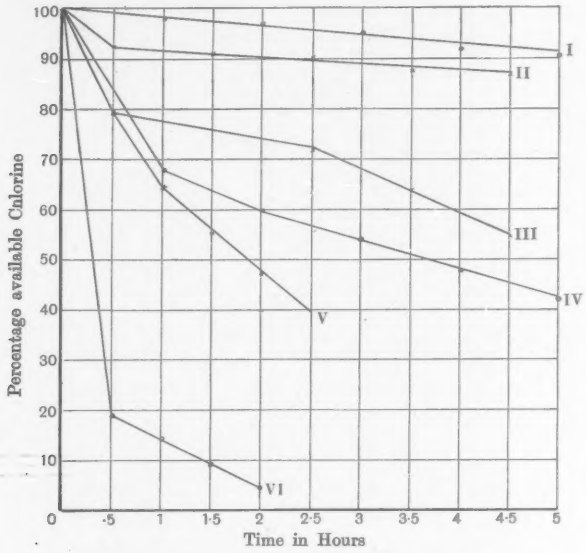
200 cc. "Eusol" (0·315% Chlorine) + 5 cc. Serum = I
„ $Mg(OCl)_2$ (0·556% „) + 9 cc. „ = II

Fig. 3.

The Action of Chloramine-T on Albumins.

The valuable work of Dakin and his collaborators [1916] has directed attention to substances containing chlorine attached to nitrogen in primary and secondary amine groups. The chemical characters and bactericidal properties of a number of aromatic chloramines prepared originally by Chattaway have been studied by these investigators. As the action of hypochlorites on albumins probably results, in small part at least, in the formation of bodies of this type, it is probable that from a comparison of the velocity of reaction of such a body as sodio-*p*-toluene-sulphochloroamide ("chloramine-T") in albuminous fluids with the reaction velocity of hypochlorites in the same fluids, one may arrive at some knowledge of the nature of the latter reaction. When chloramine solutions are mixed with varying quantities of blood serum, the reaction is not only a very much slower one but also differs in many other particulars from the hypochlorite-serum reaction. Thus the fall varies directly with the volume of serum added.

The percentage fall in available chlorine when chloramine solutions are mixed with different quantities of serum was determined in the same way as in the hypochlorite-serum mixtures. All the experiments were carried out at 36°. When small quantities of serum, similar to those used in the hypochlorite experiments, are added to chloramine solutions the rate of fall in available chlorine is extremely slow. Fig. 4 shows a series of graphs with varying concentrations of chloramine and albumin:



200 cc.	Chloramine-T	1·338 %	+	5 cc. Serum	=I
„	„	1 %	+	10 cc. „	=II
„	„	1 %	+	20 cc. „	=III
„	„	0·354 %	+	5 cc. „	=IV
„	„	0·1 %	+	10 cc. „	=V
100 cc.	„	2 %	+	100 cc. „	=VI

Fig. 4.

The fall in "available" chlorine appears to be a linear one of slow velocity usually with a short initial period with a more rapid rate of change. It is evident that with albumin concentrations such as were tested with hypochlorites the rate of fall in available chlorine is much slower in the case of chloramine and varies directly with the serum concentration. It is in the later stage of the hypochlorite-albumin reaction that one obtains a rate of

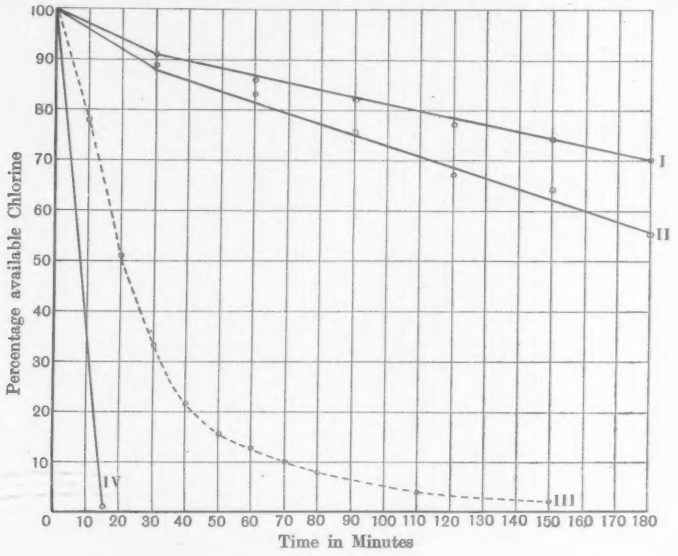
change similar to that observed with chloramine. Even with chloramine-albumin mixtures, especially in the case of the higher albumin concentrations there is also however an initial more rapid rate of change but there is not the same tendency for the chlorine to pass into the cyclic rings in the protein, as is evident from a comparison with the hypochlorite-albumin curves. It is interesting to note that with 50 % serum in a 1 % chloramine mixture, there is a rapid initial fall of a little over 80 % chlorine during the first half hour followed by a slower rate of loss, so that at the close of 2 hours the active chlorine is about 5 % of the initial concentration. Dakin, Cohen, Daufresne and Kenyon [1916] used 50 % serum concentrations for their bacteriological tests, and as they used 1–1000 to 1–2000 chloramine concentrations the available chlorine must have been brought down to an extremely low level shortly after admixture with the serum.

The Action of Hypochlorites and Chloramine-T on Amino-acids.

Langheld [1909] has studied the action of NaOCl on various amino-acids, glycine, alanine, valine, leucine etc. He found that the α-amino-acids readily react with the hypochlorite in the same way as simple amines, forming in the first place chloramino-derivatives of the acids which, on warming, readily break down forming aldehydes or ketones, ammonia, carbonic acid and sodium chloride. The products obtained from the decomposition of glycine are formaldehyde, ammonia and carbonic acid. This change takes place very rapidly. Thus when equimolecular solutions of $Mg(OCl)_2$ and glycine (1·151 g. of the former and 0·68 g. of the latter in 200 cc.) are kept at 36°, within 15 minutes over 99 % of the "available" chlorine is lost (Fig. 5 IV). A similar mixture of equimolecular chloramine and glycine, $m/20$ in each case, shows a much less rapid fall in available chlorine (Fig. 5 III). Amino-acids such as hippuric acid, which have an acid group attached to the nitrogen offer much greater resistance to the action of hypochlorite, but at 36° slow decomposition occurs as is shown in Fig. 5 I and II. Fig. 5 I shows the fall in available chlorine when 0·812 g. hippuric acid (as sodium salt) is added to 200 cc. $Mg(OCl)_2$ $m/20$ and Fig. 5 II shows the fall when $m/40$ $Mg(OCl)_2$ is taken. The nitrogen value of the added hippurate was equal to that of 5 cc. serum. The type of fall in these cases is similar to that seen in chloramine-serum reactions and differs greatly from the hypochlorite-serum reaction. Thus an increase in the hippurate-hypochlorite ratio leads

to a regular displacement downwards of the curve. Chloramine-T on the other hand shows no loss in its available chlorine even after prolonged warming with sodium hippurate.

Hypochlorite solutions therefore most readily attack the amino-acids when the N is not attached to an acid group, less readily proteins, and with greatest difficulty hippuric acid. The same order holds for chloramine-T solutions but in each case these solutions are much less effective than the



200 cc. $Mg(OCl)_2$ $m/20$ + Hippuric Acid ·812 gm. = I
" " $m/40$ + " " " = II
200 cc. containing $m/20$ Chloramine-T + $m/20$ Glycine = III
" " 1·151 gm. $Mg(OCl)_2$ + 0·68 gm. " = IV

Fig. 5.

hypochlorites. It seems most probable that the later slower fall in available chlorine occurring in protein-hypochlorite mixtures exhibits really the rate of change of chloramino-compounds formed by the action of the hypochlorite on the protein molecule.

If the germicidal action of the chloramines be a function of the available chlorine, as is probably the case, then there may be an advantage in the employment of chloramine solutions in the treatment of infected wounds when it is desired to maintain the available chlorine concentration in the

presence of albumin at a moderately high level. Thus in cases where fairly long intervals elapse between the dressing of wounds, the slower rate of change of the solutions of chloramine compared to those of hypochlorite might prove of advantage. In the primary disinfection of septic wounds, however, the greater rate at which the hypochlorite gives up its available chlorine in the attack on the protein molecule will undoubtedly result in a more efficient penetration and oxidation of necrosed material. This solvent action on necrosed tissue material is undoubtedly a valuable feature in the action of alkaline hypochlorites. The hypochlorite solutions will also act more rapidly on the products of bacterial action than the chloramine solutions of a similar strength.

Intravenous Injection of Hypochlorites and Chloramine-T.

At Professor Lorrain Smith's request the physiological effects produced by the intravenous injection of hypochlorite solutions were investigated. From the preceding study of the changes which hypochlorites undergo in albuminous solutions, one recognised that on injection into the blood there would be a very rapid fall in available chlorine taking place near the seat of injection, but there was always a possibility that the more resistant chloramine derivatives might enable the action to be sufficiently prolonged to render the blood germicidal. There was also the possibility that the powerful oxidising action of the hypochlorites might render innocuous certain products of bacterial action. This seemed much more likely than the former hypothesis, as in the cases, which have been described by Lorrain Smith and his coworkers [1915, 2], of benefit resulting from intravenous injection of comparatively small quantities of hypochlorite in the treatment of septic conditions in man, the available chlorine concentration of the blood must have been much below the germicidal level.

Hypochlorite solutions however attack the red blood corpuscles and this action must be borne in mind. Solutions of extremely low hypochlorite concentration injected in isotonic saline solution give rise to haemolysis and the pigment so set free is with greater concentrations broken down finally to colourless derivatives. The following table shows the concentrations at which haemolysis takes place when a 4 % blood corpuscle emulsion is mixed with isotonic hypochlorite and chloramine-T solutions. The tubes were left overnight at 37°.

	"Available" chlorine concentrations		
	1–20,000	1–7000	1–2500
Eusol	Very slight haemolysis	Reddish brown opaque solution	Reddish brown
Dakin's solution	,, ,,	Clear deep red solution	Pigment bleached yellow
$Mg(OCl)_2$ ...	,, ,,	,, ,, ,,	Clear deep red solution
Chloramine-T*	No haemolysis	No haemolysis	Very slight haemolysis

* (Concentrations in terms of chloramine-T not chlorine.)

On injection into the blood laking does not occur quite so readily as in the test tube experiments, but it was met with in every case of hypochlorite injection where the available chlorine concentration of the blood had been raised to 1–3000. Probably it occurs with much lower concentrations. In practically no case did chloramine injections give rise to laking. Great care requires to be taken in the case of hypochlorite injections to prevent prolonged contact of the solution with the vessel wall as there is a gradual disintegration of the lining endothelium with a possible subsequent leakage and irritation at the seat of injection. Hence as soon as the injection is finished saline ought to be used to wash away the irritant. It is advisable to use therefore a vaselined double-way cannula, so that the saline may be injected without delay after the hypochlorite.

The intravenous injections were carried out with a view to determine the point to which the available chlorine concentration of the blood might be raised without seriously affecting the circulation and respiration. The actions of eusol, Dakin's solution, $Mg(OCl)_2$ solution and chloramine-T were investigated. As the results are much the same in the case of the various hypochlorites a short general summary will be sufficient.

The experiments were carried out on rabbits, cats and in one case on the dog. When concentrations are specified, they refer to cats of approximately $2\frac{1}{2}$ kgm., unless otherwise stated.

In all cases recovery is complete when the available chlorine concentration of the blood is not raised much above 1–2000, but distinctly higher concentrations may be tolerated, for example 1–1000 or rather more. NaOCl and $Mg(OCl)_2$ may be used in slightly higher concentrations than eusol.

The first injections ought to be weaker than the later ones as the primary effects on circulation are greater than those obtained from the subsequent injections. Hypochlorite solutions containing 0·25 % "available" chlorine produce practically no disturbance until the chlorine concentration of the blood is raised to 1–1500 that is until about 40 cc. have been injected in an

animal of 3 kgm. 10 cc. of a 0·5 % solution usually give rise to an initial fall of blood pressure which is quickly recovered from and the second 10 cc. is usually without effect, but on raising the concentration to a higher level the blood pressure begins to fall.

The respirations are quickened after each injection and at least in the case of $Mg(OCl)_2$ are diminished in amplitude. Eusol in concentrations of 0·35 % or higher usually gives rise immediately after injection to slightly irregular respiratory movements. Even when hypochlorite has been added in sufficient quantity to raise the concentration in the blood to 1–600 no hypochlorite or active chlorine reaction can be obtained in a sample of blood withdrawn about half an hour after the injection. If adrenaline however be injected very shortly after hypochlorite is given, care being taken to remove the latter from the seat of injection, the usual rise in blood pressure is not obtained.

This may be due to the laked blood destroying the adrenaline, as is said to occur, or may be due to destruction by oxidation.

In all cases where the chlorine concentration is 1–2000 or more, the blood plasma after centrifugalisation is deep red in tint and the spectrum is one of oxyhaemoglobin. The coagulability of the blood is decreased. The secretion of urine owing to the saline injections is increased and in most cases it is also red in colour due to the presence of free oxyhaemoglobin.

Chloramine-T Injections.

The blood pressure is more readily affected than by hypochlorite injections. There is always a slight fall in blood pressure after the injection of 30 cc. 0·25 % chloramine into animals of an average weight of 2½ kilogrammes. When 40 cc. 0·25 % solution are injected the fall is not recovered from. This would give a chloramine concentration in the blood of 1–1250. Injections of 10–20 cc. 0·5 % chloramine and over give rise at first to a fairly rapid then a gradual fall in blood pressure which is usually not recovered from. At the same time respirations are slowed and there may be brief apnoeic periods. It is advisable therefore not to inject solutions of greater strength than 0·2 % and the chloramine concentration in the blood ought not to be raised above 1–2000. No haemolysis results from the chloramine injection of this concentration. Adrenaline injections give the usual rise of blood pressure even when given immediately after the chloramine. The coagulability of the blood is apparently not affected. Specimens of blood

withdrawn after chloramine injections do not give the reactions of active chlorine.

Dakin and his collaborators have shown that in the presence of 50 % serum the germicidal concentration of chloramine-T is approximately 1–2000. Under the conditions of possible intravenous injection in man when comparatively small quantities of chloramine would come into immediate contact with substances which would rapidly reduce the available chlorine, it is extremely unlikely that anything approaching even a temporary germicidal concentration could be attained without producing dangerous circulatory and respiratory disturbances. Intravenous injections of hypochlorites and allied substances may however prove of value in general septic conditions in some other way than by simply rendering the blood germicidal. The extremely powerful and rapid action of the hypochlorites on the proteins and most of their amino-derivatives makes it probable that a similar action will take place on the products of bacterial action.

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XXXVII. FEEDING EXPERIMENTS WITH KYNURENIC ACID.

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(*Received August 29th, 1916.*)

In the endeavour to bring to light intermediate stages in the metabolic breakdown of individual amino-acids the method of feeding an animal with substances which, on chemical grounds, represent possible intermediate products has been widely employed. If a substance so administered can, under suitable circumstances, be shown to yield a characteristic end product common to it and to the original amino-acid its claim to be a normal intermediary becomes fairly well established. The light which was thrown upon the metabolism of tyrosine and phenylalanine when Otto Neubauer administered hydroxyphenyl-lactic acid, and hydroxyphenylpyruvic acid to alcaptonuric individuals is a familiar case in point.

A product appearing in the intermediate processes of metabolism is not necessarily a degradation product in the narrower sense of the term. It may be something other than a link in the chain of processes which yield energy alone. The substance may itself subserve some function in the body, or it may be a stage in the production of a substance with a function. If an amino-acid from protein were the normal precursor of a metabolite possessed of some particular functional significance, one of the effects of withdrawing that amino-acid from the food would clearly be an interference with the function concerned. In such a case it seems probable that in the absence of the amino-acid itself the animal would benefit if an intermediate product were administered. On these lines the metabolic importance of a substance known to arise from a given amino-acid may be tested by giving it in the food in place of the parent substance. Kynurenic acid, for instance, may be fed in place of tryptophane.

A supply of tryptophane is known to be essential to the animal, which seems unable to synthesise it. While it is of course required for the formation of tissue proteins, and therefore for growth, it is probable that it also yields products with more specialised functions [Hopkins and Willcock, 1906]. Is kynurenic acid such a product, or the precursor of such a product?

Since Ellinger showed that kynurenic acid arises from tryptophane the precise significance of the appearance of a quinoline derivative during the metabolism of the indole nucleus has become a matter of interest.

Kynurenic acid might be a product on the main line of the destructive breakdown of tryptophane; it might be the end product of some accessory, non-significant, process; it might be, on the other hand, the precursor of a substance with a special function.

Miss A. Homer [1915] believes that the first possibility should be rejected, as her experiments show that (in the dog) kynurenic acid given subcutaneously, or by the mouth, is almost quantitatively excreted in the urine, though when tryptophane itself is given the amount of kynurenic acid excreted is only a small fraction of what would correspond to the metabolised tryptophane. According to Miss Homer a certain amount of the latter is side-tracked into the form of the quinoline derivative, while the greater part is broken down on other lines, or converted into substances of significance to the body.

But the fact that an intermediate metabolite is excreted more or less quantitatively when administered in excess to an animal in a state of full nutrition does not disprove the possibility that it normally arises on a significant line of change. So long as sufficient is already being produced to subserve any supposed function the excess administered may well be excreted unchanged. Only when a gap, so to speak, is first produced in metabolism by wholly withdrawing the primary source of the substance can the effect of supplying the substance itself be properly observed.

In the experiments to be described in this paper, rats were fed upon a basal dietary containing all the amino-acids of protein except tryptophane. One set of animals was fed upon this dietary alone. To the food of a second set tryptophane was added, while a third set received kynurenic acid instead of tryptophane. The results seem to show that, in the case of the rat at any rate, kynurenic acid can in no sense replace tryptophane. It does not restore to any degree the power of maintenance which a dietary always loses when deprived of tryptophane. It produces no apparent betterment in the general health of the animal, and does not lengthen the survival period.

Methods used.

The basal dietary used in the experiments had the following composition:

Amino-acid mixture obtained from prolonged acid hydrolysis of caseinogen	250 g.
Potato starch	418 „
Cane sugar	207 „
Lard	125 „
Ash of oats	13·5 „
Ash of dog biscuit	13·5 „

The fact that acid hydrolysis entirely destroys tryptophane without otherwise seriously interfering with the nutritive power of the mixture, makes the amino-acids from such hydrolysis a convenient source of tryptophane-free food. To prepare the dietary containing tryptophane 3·75 g. of the indole acid were added to the above mixture, while in the preparation of the third dietary 3·0 g. of kynurenic acid were added. Complete conversion of 3·75 g. tryptophane would yield 3·474 g. kynurenic acid. The latter was prepared from the urine of two dogs which had been given tryptophane by the mouth. It was carefully purified and melted at 89°. The necessary vitamine supply was given to the rats in the form of an alcoholic extract of the solids of fresh milk.

The animals were kept in a room of uniform temperature. They were fed and weighed at uniform intervals, and all three sets received precisely similar treatment.

Discussion of Results.

The figures give the weight curves of the rats under experiment. The first three show those of individuals, and the fourth the average weights in the case of each experimental set. The animals were fed upon bread and milk up to the 28th day (marked by an arrow) and were then put upon the experimental dietaries.

Fig. 1 shows the characteristic loss of weight which immediately follows when tryptophane is absent, the animals of this set being upon the basal dietary without any addition.

Fig. 2 shows the effect of adding tryptophane to the basal dietary. Instead of loss of weight there is maintenance, and, as seen in the average

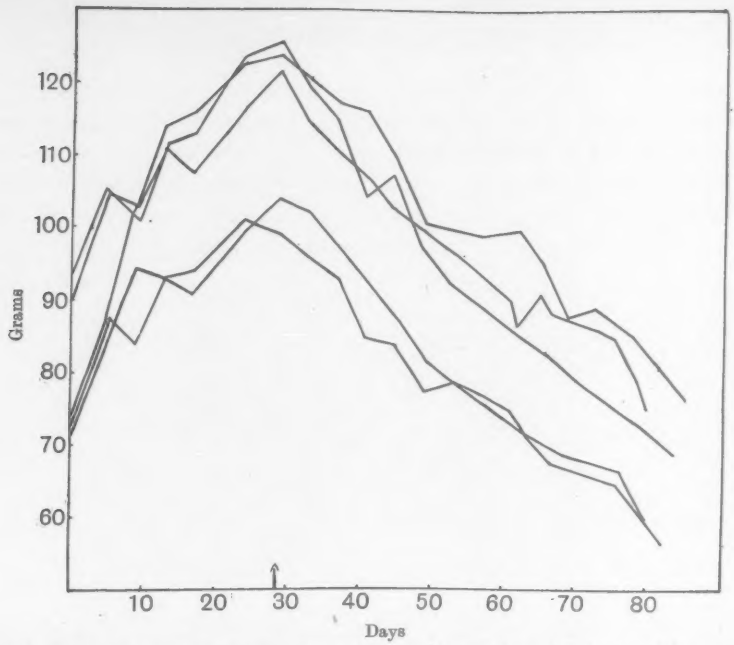

Fig. 1. Body weight curves of individual rats which from the 28th day received the basal diet alone.

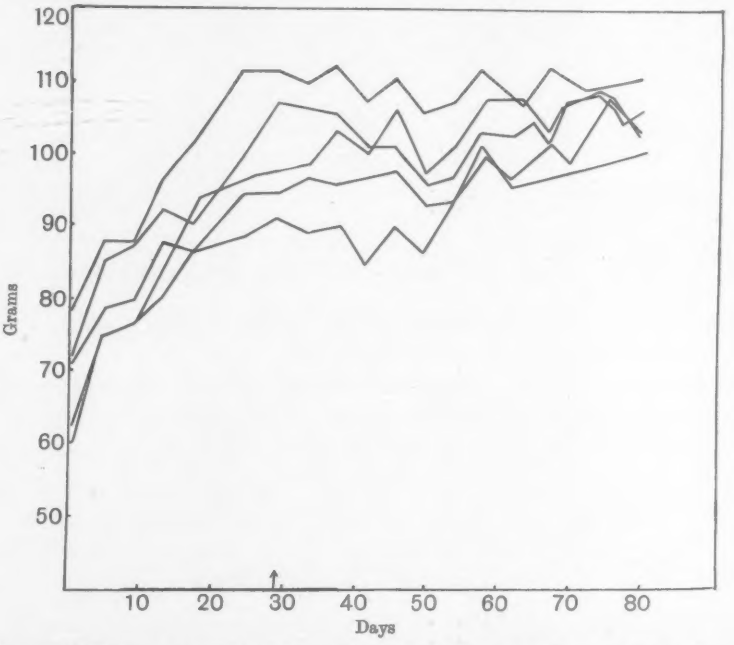

Fig. 2. Individual weight curves of rats receiving tryptophane in addition to the basal dietary.

weight curve of Fig. 4, a certain amount of growth. In a large number of experiments carried out in this laboratory, in other connections, quite normal growth has been obtained when the sole nitrogen supply was the amino-acid mixture from acid hydrolysis with added tryptophane, though a small addition of cystine has sometimes proved necessary. The somewhat less favourable result seen in my experiments was due, I think, to a deficiency in

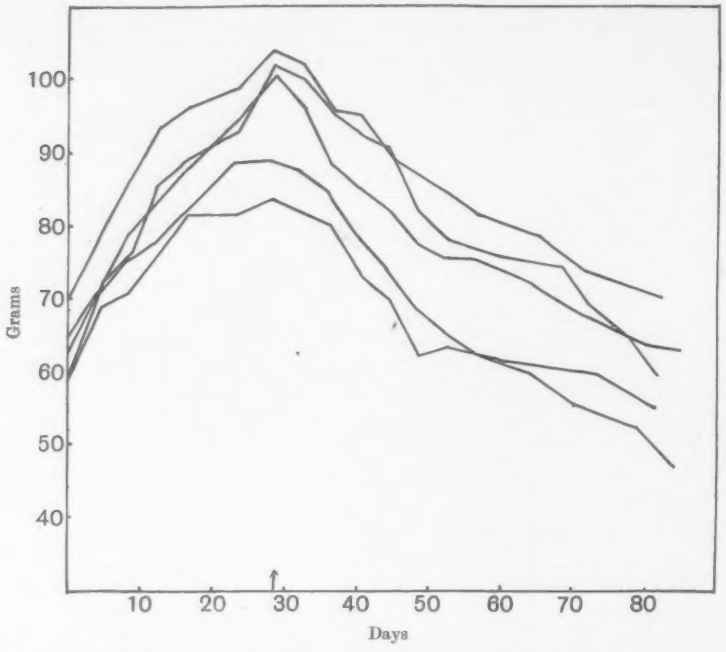

Fig. 3. Individual weight curves when kynurenic acid was added instead of tryptophane.

cystine. The protein used was caseinogen; the acid hydrolysis was prolonged (40 hours) and no cystine was added.

The effect of adding tryptophane to the basal dietary was striking enough however as will be seen from the curves. On the other hand the set of rats which received kynurenic acid instead of tryptophane showed in respect of preservation of weight no difference whatever from those on the basal dietary alone. The latter were somewhat heavier animals than the former, but it will be seen that the curves run parallel. Loss of weight occurred with equal rapidity in both. The general condition in these two sets was also strikingly

similar from week to week, and the survival period was identical, being on the average exactly fifty days in each set. In sharp contrast was the condition of the set receiving tryptophane. These rats appeared normal throughout, and were in perfect health at the end of the experiment.

The consumption of food was similar in each set and was quite satisfactory throughout. The experiments show that tryptophane cannot in any sense be replaced in the food by its quinoline metabolite. It was perhaps not to

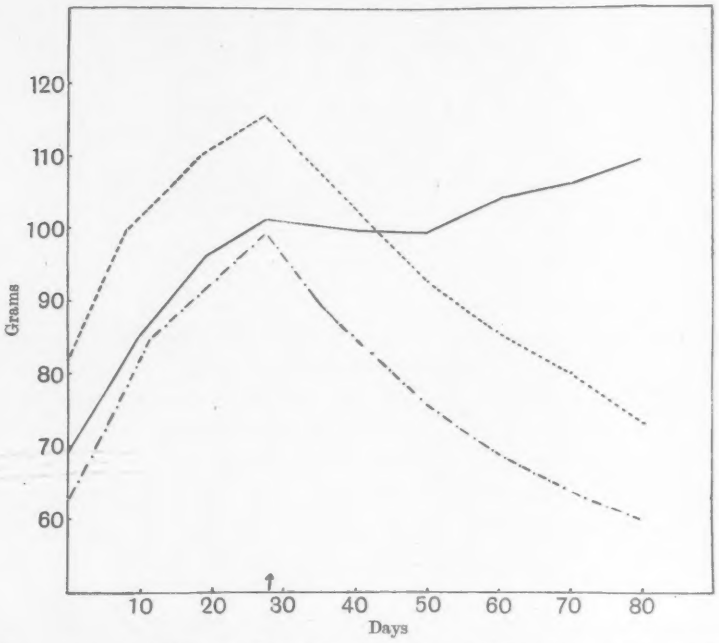

Fig. 4. Average weight curves. Dotted line, rats upon basal dietary alone. Continuous line, set upon the basal diet plus tryptophane. Dot and dash line, set taking kynurenic acid.

be expected that it could replace it as a factor in growth, as the reactions by which it is formed are not likely to be easily reversible; but, in the rat at any rate, it would appear to be without significance to the body[1].

[1] *Note by F. G. Hopkins.* Dr Asayama's results gain in significance if it be shown that kynurenic acid is a normal metabolite of the rat. The animal certainly excretes extremely little of the substance, but after feeding with tryptophane I have been able to separate small amounts of a substance from rats' urine which gives the reactions of kynurenic acid and melts at 88°–89°.

I have to thank Professor Hopkins for suggesting the experiments described, and for the interest he has taken in them. The administration of the dietaries was carried out under his direct supervision, and he has been responsible for the care of the animals.

Summary.

When tryptophane is absent from the food of rats no betterment of nutrition is observed if kynurenic acid be given. The quinoline derivative of tryptophane seems to possess no special significance in metabolism.

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XXXVIII. A STUDY OF THE DISTRIBUTION OF NITROGEN IN THE PROTEINS OF TUMOURS AND NORMAL TISSUES.

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(*Received August 15th, 1916.*)

Introductory.

A review of the literature dealing with the chemical aspects of the cancer problem will demonstrate how little serious attention, relatively speaking, this important subject has received at the hands of the biochemist.

The scattered facts which constitute our present knowledge of the subject have been ably collated by Wells [1914] in his text-book of Chemical Pathology.

Sufficient may be gathered from a perusal of his review to show that the chemist has been unable to demonstrate satisfactorily that the cancer cell is in any way strikingly different from the cell of the corresponding normal organ.

The proteins which enter into the composition of tumours have been investigated by several workers. Bang [1903] studied in particular the nucleoproteins of tumours, and was able to show that the nucleoprotein which he isolated from a lymphosarcoma was in all probability identical with the normal and characteristic nucleoprotein of lymph-glands.

Bergell and Dörpinghaus [1905] made a study of the amino-acids present in tumours, but they were unfortunately obliged to use for this investigation a somewhat mixed material from various sources. Upon analysis, using the "ester-method" of Fischer, they found that the amino-acids alanine, glutaminic acid, aspartic acid and phenylalanine were apparently present in a higher proportion than is usually found in normal tissues; while the yield of leucine, glycine and tyrosine was somewhat lower.

The analyses of Neuberg [1906], however, gave results which led him to express the view that the amounts of the amino-acids leucine, glutaminic acid, tyrosine and glycine found in tumour proteins were very much of the same order as those found in the normal organs.

Bergell [1907] investigated the proteins of human carcinomata and also of mouse cancers. Using the quantitative nitrogen-distribution method of Hausmann [1900] he obtained much higher values for the diamino-acid fraction in all cases of cancer than in normal. Thus, in human carcinoma he found that 40 % of the total nitrogen was present in the form of diamino-acid nitrogen, the figure for normal tissues being about 29 %. He also obtained similar results in the analysis of the mouse tumour proteins, and concluded that the diamino-acids played an important *rôle* in the cancer cell.

Blumenthal [1907] after having reviewed all the previous work, and also that of Wolff [1905], who found a relatively greater proportion of albumin than globulin in the press-juices of several tumours, as compared with normal tissues, concluded that important differences exist in the chemical nature of normal and malignant cells. Abderhalden and Medigreceanu [1910] examined a series of primary tumours of the liver in cows, several mouse carcinomas, and a sarcoma, quantitatively for the three amino-acids tyrosine, glycine, and glutaminic acid. Their figures show good agreement and indicate that the proportion of these substances present in tumours is not abnormal.

Wells [1914] also inclines to the view that the proteins of tumours are probably identical with, or at any rate closely similar to, those of normal organs. He explains the results of Bergell, to which allusion has been made, on the grounds that the majority of cancerous tissues are very cellular, and are therefore rich in nuclear material, and he quotes in support of this opinion the work of Petry [1899], who found that a carcinoma of the mammary gland contained a much larger proportion of nucleoprotein than did the normal gland.

We can now see that the majority of the work that has been reviewed agrees in regarding the proteins that enter into the composition of malignant growths as very similar in composition to those present in the normal organs, although we cannot consider many of the results as satisfactory from the point of view of quantitative analysis.

Recently a paper appeared by Kocher [1915], in which this author claims to have demonstrated that the amounts of the hexone bases, arginine, histidine, and lysine present in tumour proteins are approximately double those found in normal tissues. He used the method of analysis introduced by Kossel and Kütscher [1901], with the subsequent modifications recommended by

Osborne, Leavenworth and Brautlecht [1909], Steudel [1903], and van Slyke [1914]. One cannot but regard his analytical data as being somewhat insufficient, for he has only submitted five tumour proteins to analysis, and has then compared the results of these analyses with figures published some time previously by Wakeman [1908] for the diamino-acid content of normal organs. The actual tumours examined by Kocher were of a somewhat miscellaneaus nature, namely, a metastatic deposit in the liver secondary to a primary carcinoma of the stomach, a lymphosarcoma, an endothelioma, a primary carcinoma of the larynx and another secondary deposit in the liver from a primary carcinoma of the ileum.

Shortly before the appearance of Kocher's paper an examination along very similar lines was begun in this Institute as part of a detailed investigation into the chemical consideration of the cancer problem.

This examination consisted of a study of the distribution of nitrogen in the proteins of tumours, as compared with normal tissues, by the method of van Slyke.

One particular type of tumour was chosen at the outset for more or less exhaustive study, as it was realised that in order to be justified in drawing conclusions from the results obtained, it was necessary to compare examples of variations of one type of tumour—for instance the slow and rapidly growing forms—with one another, as well as with the normal organ.

The actual growth selected for study was cancer of the breast, and the reasons for this choice were several. Firstly, it is possible to obtain tumours of this variety with comparative ease, secondly, there are a large number of forms of cancer of the breast which vary in their rate of growth considerably, while for a third reason, it is possible to obtain specimens of pathological breast tissue in a condition which is regarded by many as being one of the so-called "pre-cancerous" states. On the publication of the summary of Kocher, however, the study was extended to an experimental neoplasm of the chicken which is receiving considerable attention in this Institute, namely, the Rous sarcoma.

From the summary of the paper by Kocher one is led to assume that this author regards the high values for the hexone bases in tumours as being in some way responsible for the abnormal rate of growth of these tissues, particularly in the case of the diamino-acid lysine.

It was therefore felt that this point could only be decided by an investigation of a tumour that could be controlled experimentally, and accordingly the neoplasm referred to above was chosen.

It is necessary to say that although many points remain to be decided before we can classify this particular tumour as a sarcoma, yet, for many reasons it was felt that its characters sufficiently resemble a malignant growth to justify such a study.

Indeed it would be out of place to discuss here the evidence on either side of the question as to whether the growth is one of the group of diseases known as the infective granulomata, or whether it is a form of cancer, for it is hoped that a certain amount of light on that point will be shed by the researches which are being made in this Institute at the present time.

By carefully adjusting the conditions under which this growth is propagated it is possible to vary to a considerable extent its virulence and its rapidity of growth. Originally the tumour was discovered by Rous [1910] in a hen of the Plymouth Rock strain, and for some time it was only possible to carry on the tumour by inoculating grafts when the host was a pure bred chicken of that particular breed; other strains apparently possessed a natural form of immunity. Passage of the tumour through a very large number of generations has, however, had the effect of decreasing the immunity enjoyed by the other breeds to some extent, so that if injected into a fowl of the Minorca or White Leghorn variety growth of the graft will sometimes be obtained, although usually its rate will be slow. The rate of growth in its natural host, the Plymouth Rock chicken, is generally very rapid, although even in these cases the rate is greatly influenced by the age and state of health of the host.

By a selection of suitable conditions it is therefore possible to control to some extent the rate of growth of this neoplasm; it being borne in mind that in all probability many of the conditions applied are not artificial or unnatural ones, but ones that in many cases do bear an important part in influencing the growth of human cancer. There is, unfortunately, one drawback to the use of this particular tumour for such a study as was intended, that being that it is a growth which simulates a true sarcoma in being a proliferative condition of the connective tissue of the fowl; it is therefore difficult to obtain a normal chicken tissue with which to make an adequate comparison. This point was, however, duly taken into consideration, together with the arguments in favour of using the growth, with the result that it was decided that the results of the analyses of the human tumours would serve as a control to some extent in this respect.

Experimental.

Methods of Analysis.

The proteins were in every case examined by the nitrogen distribution method of van Slyke [1911], utilising the modifications in the treatment of the phosphotungstate precipitate introduced by the same author at a later date [1915]. On arrival in the laboratory the tissues were immediately freed from all foreign tissue and finely minced in a small hand-mincing machine. The proteins of the minced tissue were then coagulated by boiling, a small quantity of dilute acetic acid being added to the boiling water. The coagulated protein was filtered off, washed with water containing a small trace of acetic acid, pressed and dried. Where necessary, traces of residual protein in solution in the filtrate were recovered by precipitation with alcohol, and added to the main portion.

The dried protein was then rendered fat-free by continuous extraction in a Soxhlet extractor with ether, after which the fat-free product was ground to a fine state of division and passed through a fine mesh sieve.

In most cases approximately 3 g. of the resulting greyish white powder was hydrolysed with 30 cc. of 20 % hydrochloric acid until hydrolysis was complete, as shown by determinations of the amino-nitrogen, the treatment of the resulting solution being then carried out along lines almost identical with those devised by van Slyke [1911].

One difficulty encountered was in the estimation of arginine, for it was found that to obtain reliable duplicate titrations was a matter of great uncertainty when the process of van Slyke was used. Plimmer [1916] has also had occasion to draw attention to this fact, and has suggested several slight modifications which undoubtedly improve the accuracy of the process.

Throughout the course of this research it has been the practice to estimate the total nitrogen of the diamino-acids fraction in a separate portion of the solution of the bases, and not in the residual liquid remaining after the determination of the arginine. Plimmer also recommends this procedure. The other improvements mentioned in his paper, namely, the use of a more dilute alkali for the hydrolysis of the arginine, and the distillation of the last traces of ammonia resulting from this hydrolysis by removing the condenser water and continuing the boiling for some twenty minutes, were adopted during the latter part of this work.

TABLE I.

Distribution of Nitrogen in the Proteins of Human Tumours.

Percentages of total nitrogen.

No. of case	Age	Weight of tumour g.	Approximate time of growth*	Characteristics of the tumour	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
331A	63	33	24 months	Scirrhus carcinoma of breast, slow growing, with hyaline degeneration of stroma and vacuolation of the protoplasm	4·42	2·24	52·20	11·80	9·45	7·50	12·39	0·70	30·04
331B	—	32	—	Rapidly growing metastatic deposits in the axillary glands from Case 331	4·72	2·47	53·30	3·75	8·40	8·10	16·44	0·96	33·90
347	63	81	6 ,,	Scirrhus carcinoma of breast, slow growing but glandular type, somewhat fibrous	5·35	1·72	57·80	11·90	10·21	7·98	4·26	1·55	24·00
342A	41	48	12 ,,	Scirrhus carcinoma of breast, slow growing with much fibrosis	3·86	2·15	50·80	12·45	9·18	6·27	14·55	—	29·90
343A	52	25	12 ,,	Scirrhus carcinoma of breast, slow growing	4·36	2·02	52·30	10·62	16·75	1·64	11·41	—	29·80
343B	—	42	—	Secondary deposits from glands of axilla from Case 343, actively growing	4·75	1·97	59·00	4·10	12·00	7·90	9·60	—	29·50
384A	57	56	6 ,,	Scirrhus carcinoma of breast, more cellular than above cases	5·87	2·84	54·90	7·40	9·51	5·77	12·72	—	28·00
384B	—	24	—	Very active metastatic deposits in glands of axilla from Case 384	3·17	2·96	59·20	4·00	9·31	7·10	14·49	—	30·90
387A	52	29	13 ,,	Scirrhus carcinoma of breast, fairly cellular and more active	6·10	2·22	51·80	8·20	11·85	6·29	11·91	1·15	31·20

387B	—	20		—	Rapidly growing secondary growths in glands from axilla from Case 387	5·03	2·50	52·90	3·42	13·10	8·00	13·67	1·03	35·80
18A	45	39	14	,,	Carcinoma of breast, alveolar type, but not rapidly growing	4·90	2·02	55·55	8·72	8·15	7·10	12·55	—	27·80
18B	—	19		—	Metastatic deposits in axillary glands from Case 18	6·19	2·49	57·80	6·37	7·90	8·80	11·60	—	28·30
332	30	87		—	Cystic mastitis, much fibrosis, no evidence of malignancy	4·57	2·57	56·89	12·89	8·69	7·23	4·80	1·28	22·00
333	19	102	24	,,	Cystic mastitis, much fibrosis, no evidence of proliferation of glandular tissue	3·01	3·09	51·10	12·43	8·91	6·44	13·55	—	28·90
349	47	84	3 weeks		General fibrosis of breast, no proliferation of glandular elements	3·74	1·39	52·70	13·70	10·75	6·86	9·20	1·20	28·01
353	49	180	3 months		General fibrosis of breast, no malignancy	3·47	1·26	51·36	11·55	13·95	6·25	10·70	1·10	32·00
20	54	60	24	,,	Myxosarcoma of breast, slow growing	4·99	1·63	53·75	9·19	12·15	6·76	10·49	0·61	30·01
97	72	25	6	,,	Adenocarcinoma of breast, fairly rapidly growing	5·76	2·29	58·80	3·83	10·90	7·00	9·90	1·60	29·40
103A	36	47	6–7	,,	Encephaloid carcinoma of breast, extending rapidly	4·19	2·42	55·40	3·03	11·10	7·75	16·24	—	35·09
103B	—	39		—	Rapidly growing secondary deposits in glands from axilla from Case 103	5·62	3·32	53·10	2·85	12·30	8·70	14·20	—	35·20
110	58	600	36	,,	Fibromyoma of uterus	5·21	1·62	52·50	10·00	9·45	7·70	12·25	—	29·40
16	20	900	6	,,	Exceedingly rapidly growing lymphosarcoma of cervical glands	4·82	4·03	50·30	2·10	11·90	8·90	16·27	1·13	38·20

* A rough approximation of the duration of growth of the tumour derived from the observations of the patients. It must be borne in mind, however, that considerable growth may have occurred before a tumour makes its presence apparent, while there is no means of ascertaining at what stage involvement of the axillary glands occur.

TABLE II.

Distribution of Nitrogen in the Proteins of the Rous Sarcoma.

Percentages of total nitrogen.

No. of case	Weight of tumour g.	Time of growth days	Characteristics of tumour	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Argin-ine N	His-tidine N	Lysine N	Cystine N	Total N of diamino-acids
5	32	30	Slow growing, somewhat fibrous	4·98	5·48	45·98	6·82	14·39	8·10	11·30	0·98	34·77
117	39	36	Somewhat fibrous small central area of mucoid degeneration	6·35	4·97	45·60	7·70	10·92	10·30	12·68	0·90	34·70
125	75	75	Very slowly growing, much fibrosis	6·25	6·25	51·30	10·10	11·94	7·59	11·05	0·82	31·40
12	32	40	Firm, slightly fibrotic tumour	4·04	5·52	43·10	8·10	11·50	11·22	13·13	0·85	36·70
3	45	42	Firm tumour, with area of central necrosis ...	6·32	6·15	48·50	7·30	11·50	9·10	10·28	0·77	31·65
90	90	30	Fairly rapidly growing tumour, with central area of mucoid degeneration	4·78	4·03	47·90	6·40	10·34	9·77	13·80	0·99	34·90
91	78	30	Firm, steadily growing tumour	5·15	1·11	48·30	6·10	11·41	10·11	12·75	1·13	35·40
133	83	21	Firm, rapidly growing, area of central necrosis	5·85	3·24	—	—	11·62	9·72	12·45	0·91	34·70
25	43	18	Fairly rapidly growing tumour, much central necrosis and mucoid change	3·98	4·95	52·40	5·70	10·72	10·00	11·90	0·78	33·40
100	70	30	Firm tumour, with some slight necrosis ...	4·68	3·66	47·70	5·60	10·44	10·12	13·48	0·66	35·70
101	68	27	Firm, solid, steadily growing tumour	6·43	3·43	48·00	5·00	10·41	10·60	13·63	1·06	35·70
72	108	18	Rapidly growing tumour, much necrosis of a myxomatous type	6·81	6·49	47·20	4·90	10·56	8·20	13·88	0·76	33·40
96	165	18	Very rapidly growing, no necrosis	6·81	6·60	50·75	4·65	11·00	10·52	10·49	0·89	32·90
139	136	36	Firm, very rapidly growing tumour	5·15	5·23	47·20	4·40	13·05	14·35	8·78	0·82	37·00
94	200	21	Rapidly growing, much central necrosis ...	6·43	5·72	50·01	4·38	12·81	8·05	10·63	0·91	32·40
94A	—	—	Metastatis in liver from 94	6·28	4·82	52·70	4·10	12·55	9·28	8·99	0·58	31·40
94B	—	—	Metastatis in lungs from 94	5·52	5·19	49·40	4·80	11·85	8·91	11·62	0·62	33·00
77	215	15	Very rapidly growing tumour, much central necrosis	5·68	4·93	46·50	4·30	11·16	12·82	12·15	0·64	36·77
60	307	51	Rapidly growing tumour, extensive central myxomatous degeneration	4·70	4·28	48·50	5·30	10·30	9·54	15·84	0·82	36·50

TABLE III.

Distribution of Nitrogen in Proteins of Normal Organs. Percentages of total nitrogen.

I. *Human.*

Organ	Remarks on organ	Amide N	Humin N	Monamino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
Pancreas ...	Normal	5·52	4·50	53·27	3·63	11·30	8·82	10·91	0·97	32·00
Kidney ...	,,	5·05	3·24	59·30	5·10	11·95	5·89	7·00	1·01	25·85
Spleen... ...	,,	5·18	3·82	47·62	4·88	12·50	13·50	11·00	1·10	38·10
Cardiac muscle	,,	5·67	1·55	52·95	5·90	9·38	10·20	12·50	0·82	32·90
Skeletal muscle	,,	6·18	2·29	58·90	4·41	11·05	2·72	13·60	0·80	28·17
Liver	,,	6·12	3·32	53·85	4·35	10·50	8·20	11·76	1·34	31·80
Cardiac muscle	,,	6·35	3·60	53·20	5·00	10·00	10·00	9·25	0·80	30·05
Kidney ...	,,	6·05	3·57	52·80	5·28	11·45	8·60	9·25	1·10	30·40
Skeletal muscle	,,	6·42	2·30	57·00	4·20	11·30	5·79	12·71	0·90	30·70
Pectoral muscle	Removed with breast No. 103	5·42	2·46	58·30	4·20	11·85	4·88	10·41	0·85	27·99
Breast... ...	Normal, but slightly fibrotic	5·50	2·50	57·17	6·40	10·10	4·66	12·47	—	27·23
,, ...	,, ,, ,,	5·10	2·85	56·20	6·00	10·02	2·81	13·38	—	26·21

II. *Chicken.*

Lungs	Normal	7·02	4·54	51·20	7·40	11·25	7·22	11·11	1·32	30·90
,, ...	,,	6·58	3·82	50·70	6·70	12·20	8·42	10·98	1·40	33·00
Liver	Stunted chick[1]	7·49	4·40	56·50	3·58	9·09	8·10	11·24	1·27	29·70
,, ...	Normal	8·19	5·56	54·80	4·95	9·15	8·00	9·25	1·30	27·70
,, ...	,,	8·17	5·52	55·20	4·00	9·45	7·44	9·45	1·16	27·50
Pectoral muscle	Stunted chick[1]	6·08	2·20	57·90	4·75	10·88	5·24	10·82	0·86	27·80
,, ,,	Normal	6·55	1·77	57·85	4·36	11·10	9·71	8·89	0·90	30·60
,, ,,	,,	6·72	1·75	56·15	4·55	11·10	7·20	10·74	0·91	29·95
Cardiac muscle	Stunted chick[1]	6·47	3·42	59·50	5·08	8·60	7·40	9·37	0·88	26·25
,, ,,	Normal	6·64	3·34	54·00	5·80	11·40	7·72	7·63	0·85	27·60
,, ,,	,,	6·02	3·17	58·05	5·10	9·99	8·00	8·36	0·90	27·25
Brain	,,	10·57	5·25	67·95		7·35	2·72	5·03	0·90	16·10

[1] Undersized chicks exhibiting signs of malnutrition through being reared from an early age indoors.

TABLE IV. *Analytical Data for*

No. of case	Total N vol. 100 cc. N in 5 cc. cc. 0·1 N acid	Total N	Amide N vol. 90 cc. cc. 0·1 N acid	Amide N	Humin N vol. 90 cc. cc. 0·1 N acid	Humin N	Monamino-acid fraction vol. 500 cc. N in 25 cc. cc. 0·1 N acid	Total N in filtrate	cc. N gas from 10 cc.	Amino N of filtrate
331A	15·00 15·15	0·4228	12·00	0·0187	6·20	0·0096	8·40 8·30	0·2600	6·62, 19·5° C., 762 mm.	0·2103
331B	14·80 14·90	0·4160	12·60	0·0196	6·65	0·0103	7·70 7·55	0·2370	7·09, 19° C., 750 mm.	0·2220
347	12·40 12·20	0·3440	11·80	0·0184	3·80	0·0059	7·60 7·80	0·2400	6·26, 19·5° C., 762 mm.	0·1985
342A	13·60 13·65	0·3810	8·40 (*k*)	0·0147	4·60	0·0082	6·70 6·85	0·2380	5·49, 16·5° C., 743 mm.	0·1935
343A	12·85 13·00	0·3612	10·10	0·0157	4·70	0·0073	7·30 7·20	0·2270	5·92, 17·5° C., 762 mm.	0·1890
343B	13·30 13·50	0·3750	13·30	0·0178	4·70	0·0073	7·70 7·55	0·2370	7·06, 19° C., 751 mm.	0·2210
384A	15·30 15·10	0·4258	16·10	0·0250	7·80	0·0121	8·55 8·50	0·2680	7·20, 15° C., 762 mm.	0·2340
384B	9·30 9·45	0·2630	5·30	0·0082	5·00	0·0078	5·30 5·30	0·1660	4·97, 16·5° C., 743 mm.	0·1656
387A	17·75 17·75	0·4973	19·45	0·0303	7·10	0·0110	9·75 9·45	0·2980	8·20, 16·5° C., 743 mm.	0·2570
387B	15·00 15·00	0·4200	13·60	0·0212	6·80	0·0105	7·60 7·60	0·2370	6·82, 15° C., 762 mm.	0·2220
18A	14·20 14·40	0·4004	12·60	0·0196	5·20	0·0081	8·20 8·30	0·2570	6·82, 15° C., 762 mm.	0·2220
18B	5·55 5·60	0·1568	6·25	0·0097	2·50	0·0039	3·25 3·24	0·1008	2·89, 24° C., 766 mm.	0·0906
332	14·60 14·80	0·4120	12·10	0·0188	6·80	0·0105	9·40 9·20	0·2900	7·20, 14·5° C., 762 mm.	0·2340
333	12·40 12·40	0·3470	5·20 (*m*)	0·0104	5·40	0·0107	5·30 5·70	0·2202	4·20, 13·5° C., 764 mm.	0·1770
349	16·90 16·90	0·4740	11·40	0·0176	3·90	0·0066	10·20 9·95	0·3145	7·92, 19° C., 750 mm.	0·2490
353	16·20 16·25	0·4540	10·10	0·0157	3·60	0·0057	9·10 9·25	0·2865	7·49, 16·5° C., 743 mm.	0·2340
20	11·80 11·90	0·3332	10·65	0·0166	3·40	0·0054	6·70 6·70	0·2090	5·72, 24° C., 766 mm.	0·1785
97	8·80 8·95	0·2492	9·20	0·0143	3·60	0·0057	5·00 5·00	0·1560	4·66, 24° C., 766 mm.	0·1460
103A	16·35 16·05	0·4540	12·20	0·0190	7·10	0·0110	8·55 8·45	0·2650	7·91, 18·5° C., 765 mm.	0·2520
103B	16·00 15·85	0·4452	16·10	0·0250	9·50	0·0148	8·00 8·00	0·2490	7·48, 18·5° C., 765 mm.	0·2370
110	17·50 17·70	0·4930	16·40	0·0256	5·10	0·0080	9·90 9·85	0·3080	8·13, 18·5° C., 765 mm.	0·2590
16	15·80 15·60	0·4400	13·60	0·0212	11·45	0·0177	7·50 7·30	0·2310	7·05, 21·5° C., 760 mm.	0·2220

(*k*) Estimations carried out on 80 cc.
(*m*) Estimations carried out on 70 cc.

Proteins of Human Tumours.

		Diamino-acid fraction vol. 50 cc.						
N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.	Amino N of bases	Arginine N in 10 cc. cc. 0·1 N acid	Argin-ine N	g. $BaSO_4$ from 5 cc.	Cystine N	Total N re-covered
8·30 / 8·20	0·1300	11·90, 15° C., 765 mm.	0·0763	2·60	0·0405	0·0009	0·0030	0·4183
9·10 / 9·10	0·1410	12·22, 15° C., 765 mm.	0·1018	2·30	0·0350	0·0012	0·0040	0·4079
5·40 / 5·20	0·0825	6·15, 15° C., 765 mm.	0·0397	2·25	0·0350	0·0016	0·0053	0·3468
6·55 / 6·65	0·1145	10·20, 17° C., 750 mm.	0·0729	2·00	0·0350	—	—	0·3754
6·90 / 6·80	0·1070	8·90, 15° C., 765 mm.	0·0581	3·90	0·0605	—	—	0·3570
7·10 / 7·10	0·1105	8·75, 15° C., 765 mm.	0·0570	2·90	0·0450	—	—	0·3726
7·65 / 7·70	0·1200	11·30, 15° C., 765 mm.	0·0725	2·60	0·0405	—	—	0·4251
5·20 / 5·20	0·0810	7·80, 15° C., 765 mm.	0·0510	1·60	0·0245	—	—	0·2630
9·90 / 9·85	0·1545	14·12, 22° C., 760 mm.	0·0889	3·80	0·0590	0·0017	0·0057	0·4828
9·65 / 9·70	0·1510	13·90, 22° C., 760 mm.	0·0873	3·52	0·0550	0·0013	0·0043	0·4180
7·20 / 7·10	0·1110	10·60, 21° C., 753 mm.	0·0669	2·10	0·0325	—	—	0·3957
2·85 / 2·85	0·0444	4·00, 15·5° C., 765 mm.	0·0258	0·80	0·0124	—	—	0·1587
5·70 / 5·85	0·0905	6·90, 17° C., 750 mm.	0·0436	2·30	0·0357	0·0016	0·0053	0·4098
4·85 / 5·00	0·1000	5·94, 17° C., 750 mm.	0·0509	1·55	0·0310	—	—	0·3113
8·50 / 8·50	0·1325	11·25, 15° C., 765 mm.	0·0724	3·35	0·0510	0·0017	0·0057	0·4712
9·30 / 9·20	0·1445	11·50, 15° C., 765 mm.	0·0772	4·10	0·0635	0·0015	0·0050	0·4524
6·50 / 6·35	0·1000	8·70, 21·5° C., 757 mm.	0·0546	2·65	0·0405	0·0006	0·0020	0·3310
4·70 / 4·70	0·0730	5·95, 21·5° C., 757 mm.	0·0389	2·20	0·0272	0·0012	0·0040	0·2490
10·22 / 10·20	0·1590	15·70, 22° C., 758 mm.	0·0972	3·25	0·0505	—	—	0·4541
10·00 / 10·20	0·1570	14·25, 22° C., 758 mm.	0·0894	3·55	0·0550	—	—	0·4458
9·20 / 9·40	0·1450	13·20. 18·5° C., 765 mm.	0·0818	2·97	0·0465	—	—	0·4866
10·70 / 10·85	0·1680	16·05, 21° C., 753 mm.	0·1061	3·40	0·0525	0·0015	0·0050	0·4379

TABLE V. *Analytical Data for*

No. of case	Total N vol. 100 cc. N in 5 cc. cc. 0·1 N acid	Total N	Amide N vol. 95 cc. cc. 0·1 N acid	Amide N	Humin N vol. 95 cc. cc. 0·1 N acid	Humin N	Monamino-acid fraction vol. 200 cc. N in 25 cc. cc. 0·1 N acid	Total N in filtrate	cc. N gas from 10 cc.	Amino N of filtrate
5	15·65	0·4385	14·60	0·0215	16·30	0·0240	19·65	0·2320	17·00, 20°, 752	0·2020
117	16·00 (*a*)	0·4480	18·25	0·0284	14·30	0·0222	19·00	0·2360	15·70, 18·5°, 770	0·2037
125	14·90	0·4178	17·70	0·0261	17·70	0·0261	8·65 (*c*)	0·2550	8·98, 18°, 766	0·2133
12	14·30	0·4010	11·00	0·0162	15·05	0·0222	18·10	0·2140	14·60, 20°, 752	0·1730
3	14·30	0·4010	17·20	0·0254	16·80	0·0247	7·60 (*c*)	0·2240	6·35, 17·5°, 764	0·1940
90	14·50	0·4070	13·20	0·0195	11·10	0·0164	18·75	0·2210	16·10, 16°, 754	0·1950
91	15·50	0·4348	15·20	0·0224	3·25	0·0048	20·10	0·2360	17·30, 16°, 754	0·2100
133	16·90 (*b*)	0·4730	16·80	0·0277	9·30	0·0153	—	—	—	—
25	16·05 (*a*)	0·4495	11·70	0·0179	14·30	0·0223	17·55	0·2365	18·85, 18·5°, 770	0·2355
100	13·60	0·3820	12·10	0·0179	9·50	0·0140	17·35	0·2040	15·20, 18°, 750	0·1819
101	14·40	0·4040	17·50	0·0258	9·30	0·0137	18·10	0·2140	16·20, 18°, 750	0·1935
72	14·50	0·4070	18·60	0·0274	17·90	0·0264	7·20 (*c*)	0·2120	6·28, 17·5°, 764	0·1920
96	14·80	0·4150	19·20	0·0283	16·80	0·0274	7·80 (*c*)	0·2300	6·88, 17·5°, 764	0·2100
139	14·80	0·4150	14·50	0·0214	14·70	0·0217	18·10	0·2140	16·50, 20°, 752	0·1960
94	15·80	0·4425	19·30	0·0285	17·10	0·0253	8·20 (*c*)	0·2410	7·45, 19·5°, 757	0·2220
94A	15·47	0·4335	18·40	0·0272	14·10	0·0208	8·35 (*c*)	0·2460	7·68, 20°, 757	0·2290
94B	14·40	0·4040	15·10	0·0223	14·20	0·0209	7·40 (*c*)	0·2180	6·65, 18°, 754	0·1990
77	16·90 (*a*)	0·4730	17·20	0·0268	15·00	0·0233	19·35	0·2410	16·92, 18·5°, 771	0·2200
60	22·20	0·6210	19·80	0·0292	18·08	0·0266	28·35	0·3350	24·00, 20°, 752	0·3070

(*a*) Volume of liquid carried on for analysis 90 cc.
(*b*) Volume of liquid carried on for analysis 85 cc.
(*c*) Volume of monamino-acid fraction 500 cc.

In spite of these improvements in the technique of this particular estimation, it has been found difficult to obtain concordant results. The figures for arginine are therefore not regarded as being more than approximately accurate. There is reason to believe that the values given for this amino-acid are lower than they should be, and consequently those for histidine are somewhat high.

Corrections for the solubilities of the phosphotungstates of the basic amino-acids have not been applied as results of comparative value were alone required.

The human tumour tissues utilised in this work were in all cases obtained from the Pathological Department of the Cancer Hospital. The majority of these cases were specimens removed at operation, and were in all cases available for analysis from 6 to 12 hours subsequently. One or two of the tissues were obtained post-mortem, and in these cases the time elapsing between death and the treatment of the tumour tissue was usually somewhat longer.

Proteins of Rous Sarcoma.

		Diamino-acid fraction vol. 50 cc.						
N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.	Amino N of bases	Arginine N in 10 cc., cc. 0·1 N acid	Arginine N	g. $BaSO_4$ from 5 cc.	Cystine N	Total N recovered
10·30	0·1520	13·50, 18·6° C., 752 mm.	0·0805	4·30	0·0632	0·0015	0·0043	0·4295
10·00	0·1558	13·65, 18·5° C., 771 mm.	0·0883	3·15	0·0490	0·0015	0·0040	0·4424
8·90	0·1310	12·00, 18° C., 750 mm.	0·0715	3·40	0·0500	0·0012	0·0034	0·4382
10·05	0·1475	12·10, 18·5° C., 756 mm.	0·0727	3·30	0·0462	0·0012	0·0034	0·3999
9·60	0·1290	11·60, 19° C., 761 mm.	0·0700	3·30	0·0462	0·0011	0·0031	0·4031
9·65	0·1415	13·85, 19° C., 761 mm.	0·0837	2·85	0·0420	0·0014	0·0040	0·3984
10·40	0·1535	14·39, 19° C., 761 mm.	0·0869	3·37	0·0496	0·0017	0·0048	0·4167
9·95	0·1640	13·45, 17·5° C., 766 mm.	0·0919	3·35	0·0591	0·0017	0·0043	—
9·60	0·1490	12·80, 18·5° C., 772 mm.	0·0829	3·10	0·0483	0·0013	0·0035	0·4257
9·28	0·1632	13·10, 18·5° C., 772 mm.	0·0804	2·72	0·0400	0·0009	0·0025	0·3991
9·78	0·1440	13·63, 18·5° C., 772 mm.	0·0837	2·86	0·0422	0·0015	0·0043	0·3975
9·20	0·1356	13·45, 19·5° C., 760 mm.	0·0810	2·92	0·0430	0·0011	0·0031	0·4014
9·30	0·1376	12·40, 19·5° C., 760 mm.	0·0745	3·10	0·0456	0·0013	0·0037	0·4233
10·40	0·1535	12·30, 19·5° C., 752 mm.	0·0732	3·67	0·0541	0·0012	0·0034	0·4106
9·70	0·1435	13·10, 19° C., 745 mm.	0·0772	3·85	0·0568	0·0014	0·0040	0·4383
9·20	0·1355	11·50, 19° C., 745 mm.	0·0678	3·70	0·0545	0·0009	0·0025	0·4295
9·00	0·1330	12·38, 19° C., 745 mm.	0·0730	3·25	0·0479	0·0009	0·0025	0·3942
11·18	0·1740	14·55, 18·5° C., 767 mm.	0·0938	3·40	0·0528	0·0011	0·0030	0·4651
15·40	0·2270	23·40, 19·5° C., 752 mm.	0·1392	4·35	0·0641	0·0018	0·0051	0·6178

It was found somewhat difficult to obtain accurate information as to the length of time during which these tumours had been growing—a point of some importance in this investigation—and it has therefore been necessary to rely on the approximate data furnished by the patients themselves. In this respect I must state my indebtedness to the Medical Staff of the Cancer Hospital for their kindness in placing at my disposal the clinical records of the cases examined. For all but three of the normal human tissues I must express my gratitude to Dr E. H. Kettle, Assistant Pathologist to St Mary's Hospital, London. They were obtained from normal subjects who had been the victims of fatal accidents.

The Rous tumours were obtained by experimental inoculation of small grafts of the tumour made in the pectoral muscle of a series of fowls. The subsequent development of the graft was then closely watched and all the particulars of its growth carefully noted. By this means it was possible to gain possession of much valuable data with regard to the growth of each individual tumour which it was seldom possible to get in the human cases.

The full particulars of the cases are not reproduced in entirety, but the points of actual importance with regard to each tumour are given in the tables so that they are in the best position for purposes of comparison.

The normal chicken organs were taken from healthy adult hens of the Plymouth Rock strain. Included in the tables of the analyses of these normal chicken organs will be seen several which are described as being taken from stunted chicks; these chicks were ones which had been reared from an early age in this Institute and which had become stunted before reaching the adult age [Drummond, 1916].

Discussion of Results.

From a consideration of the analytical data recorded in the tables (I–VI), it is quite apparent that this research has entirely failed to corroborate the conclusions of Kocher. That this is so will be more easily realised if the following table is surveyed. It contains the average figures for each group of analyses made in this study compared with the averages given by him, from which he draws his conclusions.

TABLE VII.

Average Values for Distribution of Nitrogen in Proteins of Normal and Cancer Tissues.

	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of diamino-acids
Human tumours ...	4·73	2·42	54·24	7·90	10·09	7·09	12·04	1·12	30·54
Normal human tissues	5·82	3·09	55·88	4·94	11·13	7·86	9·83	0·93	30·71
Chicken tumour ...	5·58	4·84	48·44	5·87	11·50	9·96	12·04	0·83	34·38
Normal chicken organs	7·20	3·73	55·62	5·11	10·13	7·26	9·40	1·05	27·70
Normal organs (*a*) ...	—	—	—	—	6·07	1·78	5·68	—	13·52
Human tumours (*b*)	—	—	—	—	12·42	4·86	11·23	—	28·48

(*a*) consists of the averages of a series of analyses of normal tissues made by Wakeman [1908], and quoted by Kocher [1915], for purposes of comparison with the results of the analyses of five tumour proteins made by the latter author, which are averaged at (*b*).

The normal tissues which are included in the average (*a*), however, include widely diverse organs taken from man, dog, ox, and horse.

It is also at once apparent that the great error made by Kocher was in not carrying out control estimations upon normal human tissues himself, for his results for the partition of nitrogen in the tumour proteins are of very much the same order as those which have been obtained during the course of this research. His figures for the percentages of arginine and histidine are probably more correct than the ones given in this communication, because in several cases his determinations were controlled by the isolation of these

TABL

Analytical Data for Proteins

Tissue	Total N vol. 100 cc. N in 5 cc. cc. 0·1 N acid	Total N	Amide N vol. 90 cc. cc. 0·1 N acid	Amide N	Humin N vol. 90 cc. cc. 0·1 N acid	Humin N	Monamino-acid fraction vol. 500 N in 25 cc. cc. 0·1 N acid	Total N in filtrate	cc. N gas from 10 cc
Pancreas 1 ...	14·15 14·30	0·3980	14·10	0·0219	11·50	0·0179	7·30 7·30	0·2265	7·20, 20° C., 751 m
Kidney 1 ...	15·00 15·10	0·4220	13·60	0·0212	8·80	0·0137	8·70 8·70	0·2707	8·00 8·00 22° C., 758 m
Spleen 1	15·95 16·10	0·4480	14·90	0·0232	9·95	0·0155	7·50 7·60	0·2350	7·10 7·05 22° C., 758 m
Cardiac muscle 1	14·40 14·15	0·4000	14·70	0·0227	3·95	0·0062	7·50 7·50	0·2350	6·80 6·75 22° C., 758 m
Skeletal muscle 1	14·40 14·55	0·4050	16·10	0·0250	6·00	0·0093	8·25 8·30	0·2575	8·06, 20° C., 751 m
Cardiac muscle 2	15·00 15·00	0·4200	17·20	0·0267	9·70	0·0151	7·85 7·90	0·2445	7·10 7·15 20° C., 751 m
Liver 2	12·95 12·90	0·3620	14·20	0·0221	7·70	0·0120	6·80 6·75	0·2100	6·42, 20° C., 764 m
Kidney 2 ...	14·50 14·60	0·4075	15·80	0·0246	9·40	0·0146	7·60 7·60	0·2360	6·75 6·75 20° C., 764 m
Skeletal muscle 2	17·30 17·30	0·4840	20·00	0·0312	7·10	0·0111	9·55 9·55	0·2960	9·32, 20° C., 751 m
Pectoral muscle 3	15·20 15·20	0·4260	14·80	0·0231	6·70	0·0104	8·65 8·50	0·2660	8·25, 20° C., 764 m
Breast 4	14·00 13·85	0·3900	13·80	0·0215	6·30	0·0098	8·00 8·02	0·2480	7·40, 20° C., 764 m
Breast 5	14·00 14·00	0·3925	12·85	0·0200	7·20	0·0112	7·85 7·90	0·2446	7·35, 20° C., 764 m

Normal Chick

Lungs 3	13·80	0·3860	*18·40	0·0272	*11·90	0·0175	7·70	0·2260	6·50, 20° C., 772 mm
Lungs 2	14·88	0·4160	18·60	0·0274	10·80	0·0159	8·10	0·2385	6·93, 20° C., 770 mm
Liver 1	13·65	0·3820	19·50	0·0287	11·40	0·0168	7·80	0·2295	7·11, 20° C., 772 mm
Liver 2	11·55	0·3240	18·10	0·0267	12·30	0·0181	6·60	0·1940	5·85, 20° C., 770 mm
Liver 3	11·70	0·3270	18·25	0·0269	12·30	0·0181	6·60	0·1940	5·95, 20° C., 772 mm
Pectoral muscle 1	10·20	0·2860	11·80	0·0174	4·27	0·0063	6·05	0·1780	5·45, 20° C., 772 mm.
Pectoral muscle 2	15·42	0·4320	19·20	0·0283	4·90	0·0072	8·80	0·2595	8·25, 20° C., 770 mm.
Pectoral muscle 3	15·90	0·4455	20·30	0·0299	5·30	0·0078	9·20	0·2705	8·25, 20° C., 772 mm.
Cardiac muscle 1	7·85	0·2195	9·60	0·0142	5·10	0·0075	4·80	0·1415	4·30, 22° C., 771 mm.
Cardiac muscle 2	14·45	0·4050	18·20	0·0268	9·10	0·0134	8·20	0·2420	7·20, 20° C., 772 mm.
Cardiac muscle 3	9·30	0·2605	10·70	0·0158	5·60	0·0082	5·60	0·1647	4·97, 20° C., 770 mm.
Brain	9·30	0·2605	18·70	0·0276	9·30	0·0137	6·00	0·1765	—

* Volume analysed 95 cc. in all this series.

ABLE VI.

ins of Normal Human Tissues.

. 500 cc.		Diamino-acid fraction vol. 50 cc.									
10 cc.	Amino N of filtrate	N in 5 cc. cc. 0·1 N acid	Total N of bases	cc. N gas from 5 cc.		Amino N of bases	Arginine N in 10 cc. cc. 0·1 N acid	Arginine N	g. $BaSO_4$ from 5 cc.	Cystine N	Total N recovered
751 mm.	0·2125	8·15 8·20	0·1270	10·90 11·00	19·5° C., 763 mm.	0·0700	2·90	0·0450	0·0011	0·0037	0·3933
758 mm.	0·2500	7·00 6·95	0·1090	8·00 7·75	20° C., 764 mm.	0·0547	3·25	0·0505	0·0012	0·0040	0·4046
758 mm.	0·2190	11·20 11·30	0·1750	14·55 14·60	19·5°C., 763 mm.	0·0928	3·62	0·0560	0·0015	0·0049	0·4487
758 mm.	0·2110	8·40 8·50	0·1315	13·50 13·60	19·5° C., 763 mm.	0·0862	2·42	0·0375	0·0010	0·0033	0·3954
751 mm.	0·2380	7·55 7·65	0·1180	12·20 12·30	19·5° C., 763 mm.	0·0772	2·87	0·0446	0·0010	0·0033	0·4098
751 mm.	0·2240	8·10 8·10	0·1260	10·40 10·44	19·5° C., 763 mm.	0·0665	2·70	0·0420	0·0010	0·0033	0·4123
764 mm.	0·1940	7·15 7·10	0·1115	10·40 10·40	20° C., 764 mm.	0·0663	2·45	0·0380	0·0014	0·0047	0·3556
764 mm.	0·2145	7·95 7·95	0·1239	10·30 10·20	21° C., 764 mm.	0·0653	3·04	0·0466	0·0013	0·0044	0·3991
751 mm.	0·2760	9·60 9·55	0·1485	14·30 14·00	20° C., 764 mm.	0·0889	3·50	0·0545	0·0013	0·0044	0·4868
764 mm.	0·2490	7·95 8·00	0·1240	11·30 11·40	20° C., 764 mm.	0·0722	3·25	0·0505	0·0011	0·0037	0·4235
764 mm.	0·2230	6·80 6·80	0·1060	10·10 10·00	21° C., 764 mm.	0·0644	2·53	0·0394	—	—	0·3853
764 mm.	0·2210	6·60 6·60	0·1030	10·39 10·30	21° C., 764 mm.	0·0660	2·53	0·0394	—	—	0·3788

Chicken Tissues.

772 mm.	0·1975	8·15 8·10	0·1195	11·30 11·40	19·5° C., 762 mm.	0·0684	2·94	0·0434	0·0016	0·0051	0·3902
770 mm.	0·2105	9·40 9·25	0·1370	12·60 12·40	19·5° C., 762 mm.	0·0758	3·43	0·0508	0·0018	0·0058	0·4188
772 mm.	0·2160	7·80 7·60	0·1135	11·30 11·30	18° C., 742 mm.	0·0670	2·35	0·0346	0·0015	0·0049	0·3885
770 mm.	0·1775	6·00 6·25	0·0900	8·30 8·40	19° C., 762 mm.	0·0506	2·00	0·0295	0·0013	0·0041	0·3288
772 mm.	0·1810	6·00 6·25	0·0900	8·30 8·35	17·5° C., 772 mm.	0·0505	2·10	0·0310	0·0012	0·0038	0·3290
772 mm.	0·1655	5·40 5·40	0·0795	7·50 7·45	17·5° C., 772 mm.	0·0461	2·11	0·0311	0·0008	0·0025	0·2812
770 mm.	0·2500	9 55 9·55	0·1405	12·30 12·50	17·5° C., 772 mm.	0·0765	3·26	0·0479	0·0012	0·0038	0·4355
772 mm.	0·2500	9·00 9·10	0·1335	13·80 13·80	18° C., 742 mm.	0·0834	2·60	0·0383	0·0013	0·0041	0·4417
771 mm.	0·1302	3·90 3·90	0·0575	4·55 4·60	18° C., 742 mm.	0·0280	1·10	0·0250	0·0006	0·0019	0·2207
772 mm.	0·2185	7·70 7·55	0·1120	9·10 9·30	19° C., 761 mm.	0·0565	3·14	0·0461	0·0011	0·0038	0·3942
770 mm.	0·1510	4·80 4·85	0·0710	7·20 7·25	18·5° C., 762 mm.	0·0436	1·77	0·0260	0·0007	0·0022	0·2597
	—	2·82 2·85	0·0416	3·70 3·75	18·5° C., 762 mm.	0·0225	1·30	0·0191	0·0007	0·0022	0·2594

substances in the form of their picrolonates. It will be noticed that his values for arginine are somewhat higher, and those for histidine slightly lower than have been found in the cases recorded here; this, however, is explainable by the error which it is believed exists in the determinations of the first-named substance.

The averages given in the last table are only of value to indicate whether any striking difference exists between tumour tissues and normal tissues from the point of view of the present study, for they are not in many cases strictly comparable; thus, for example, it is not permissible to draw any definite conclusion from the comparison of the amino-acid partition of the proteins of a sarcoma and of normal voluntary muscle, for we are dealing with two totally different types of tissue. We must therefore draw our conclusions as far as possible from the comparison of the normal and malignant cell of the same type.

Owing to the great difficulty of obtaining many of the specimens one would like to examine, the possibilities of carrying this out are very restricted, and only in the case of one organ—the breast—has this ideal comparison been to some extent possible. Even in this case the ideal has, unfortunately, not been realised to the extent hoped for, owing to the difficulty in obtaining certain of the material, particularly the normal gland (Table VIII).

TABLE VIII.

Average Values for the Distribution of Nitrogen in the Proteins of Normal and Pathological Conditions of the Mammary Gland.

	Amide N	Humin N	Mon-amino N of filtrate	Non-amino N of filtrate	Arginine N	Histidine N	Lysine N	Cystine N	Total N of bases
Normal	5·30	2·67	56·68	6·20	10·06	3·73	12·92	—	26·72
General fibrosis ...	3·94	2·08	53·01	12·64	10·58	6·69	9·56	1·19	27·72
Slow growing scirrhus	4·49	2·03	53·30	11·69	11·42	5·84	10·65	1·10	28·46
Rapidly growing tumours	5·36	2·35	55·29	6·23	10·30	5·78	12·06	—	30·30
Secondary deposits in glands	4·91	2·62	55·88	4·08	10·50	8·10	13·33	—	32·60

Coming to a more detailed study of the various groups of analytical data we will first turn to the results obtained in an examination of the normal and pathological mammary gland.

Only two normal breasts have been examined, owing to the difficulty mentioned, and in both these cases there was present a slight degree of fibrosis.

Fibrosis of the breast when extensive may, and often does, give rise to consequences which make the removal of the gland advisable; in fact the condition is regarded by many as being one of the so-called "pre-cancerous" states. If this view is adopted one may fairly compare results obtained in the analysis of such a case with the normal breast on the one hand, and with the case of the fibrous scirrhus carcinoma of the breast on the other. Four examples of this excessive fibrosis of the breast have been investigated. The average percentage of nitrogen present in the form of diamino-acids is 27·72, a figure that is slightly higher than the corresponding value of 26·92 for the normal gland. The large amount of fibrous tissue present in the cases of so-called chronic mastitis is to some extent reflected in the abnormally high value for the proline group of amino-acids, which averages 12·64 % of the total nitrogen, as compared with the figure 6·2 % in the case of the protein of the normal gland.

A similarly high percentage for the nitrogen present in this form is also recorded in the analysis of the uterine fibromyoma, another structure very rich in fibrous tissue. In the examples of the slow growing scirrhus carcinomata of the breast, of which there are five, we have a tissue which besides showing a certain degree of proliferation of the glandular elements, also possesses a considerable increase in the amount of fibrous stroma. Both these facts are distinctly traceable in the analytical results; thus the diamino-acid nitrogen percentage of 28·13 is a higher figure than that recorded for either the normal gland or for the mastitic condition, while the comparatively high figure for the proline group of amino-acids denotes a larger amount of the proteins of fibrous tissue than is normal. Taking now the more rapidly growing tumours of the organ under examination, examples of which are the encephaloid carcinomata, the adenocarcinoma, and the myxosarcoma, we find that the percentage of the diamino-acids is somewhat larger, 30·92 % of the total nitrogen being present in this form, whilst the proportion of the non-amino-acid of the filtrate is considerably lower.

Once again both these results are in accordance with the fact that we are now dealing with the more cellular type of tumour, that is one richer in nuclear material and relatively poorer in connective tissue stroma.

As is well known, malignant tumours of the breast are particularly prone to form metastatic deposits in the lymphatic glands of the axilla, and it is probable that in some cases the rate of growth of these secondary deposits is relatively much more rapid than that of the primary growth, especially in the case of the slow growing scirrhus tumours, a result that

is possibly due to what may be called the greatly improved environment of the secondary foci.

Six specimens of such secondary deposits have been analysed, and in each case it is possible to compare these analyses with those of the primary growths from which they are derived. The results are uniform in nature, while their average will show that their greater rate of growth, and therefore their greater content of cellular matter, is portrayed in both the high figure of 32·36 % for the diamino-acid nitrogen, and the low value of 4·08 % for the proline fraction.

The other tumours which have been analysed have given results of both interest and importance. Case No. 16 is particularly interesting as being an exceedingly rapidly growing lymphosarcoma of the cervical glands. Occurring in a young man of twenty, it attained a very large size and weighed over 900 g. in the unusually short space of six months. Microscopically the tumour was seen to be an extremely active one, containing a very large amount of nuclear substance, with little supporting tissue, and this observation is well borne out by the analytical data. The percentage of the total nitrogen present in the form of diamino-acids in this tumour is the highest recorded in the whole series of analyses, namely, 38·20. It is also of considerable interest to compare this analysis with the only analysis made upon a normal lymph-gland, that of the spleen, when it will be seen that in this normal organ we have an almost identical figure for this determination, namely, 38·10 %. Results such as these should have much weight in effectively disposing of the theory that in cancer tissues we have an abnormally high proportion of the diamino-acids.

Turning now to the analytical data recorded for the proteins of the Rous sarcoma and normal chicken tissues, we find that they are very similar in nature to those we have just considered. As has been already remarked, we are not strictly justified in drawing comparisons between these two types of tissues because no normal example of the connective tissue of the fowl has been examined; for it must be remembered that the neoplasm under consideration is one of connective tissue origin.

From the average figures of analysis it will be seen that there is a distinctly higher proportion of the diamino-acids present in the proteins of the tumours of this series than in the normal chicken tissues. This is in all probability due to the much more cellular character of the tumours, for microscopically the growth is a richly cellular structure possessing a very large amount of nuclear substance.

By the study of this particular neoplasm it was hoped that it would be possible to trace in the results the influence of the rate of growth upon the content of diamino-acids. From the summarised results given below, however (Table IX), it will be seen that this expectation has not been realised to the extent that was desired. Differences of a small nature do exist, and they are in every case in the direction which was expected, but it is probably due to the fact that the slowest growing tumour of this type is relatively rich in nuclear matter that they are not of a more striking order.

TABLE IX.

Influence of the Rate of Growth upon the Distribution of Nitrogen in the Proteins of the Rous Sarcoma.

Rate of growth[1]	Amide N	Humin N	Mon-amino N	Non-amino N	Arginine N	Histidine N	Lysine N	Cystine N	Total diamino N
Approx. 1 g. per day	5·57	5·67	46·89	8·00	12·05	9·26	11·68	0·86	33·83
From 2–4 g. per day	5·14	3·40	48·86	4·90	10·82	10·05	13·00	0·92	34·90
From 5–12 g. per day	5·93	5·38	48·38	4·66	11·48	10·58	11·96	0·81	35·79

[1] An approximation derived from the final weight of the excised primary growth and the period of time from the injection to the excision. The weight of tumour tissue in the bird in the form of metastatic deposits is not allowed for, although in some cases considerable.

From a general survey of the analyses as a whole, it must be acknowledged that there is more evidence to be derived from them in favour of the opinion that the higher proportion of hexone bases in malignant growths is dependent on the amount of nuclear substance present, than there is in support of the view, held by some, that this fact constitutes a specific property of the cancer cell.

Turning now to the individual amino-acids which have been estimated, it is felt that the accuracy of these estimations is not of a sufficiently high degree to warrant any definite deductions. On the other hand, if it is assumed that the errors of determination are of such a character that the results yielded by them may be used for a roughly comparative study, then one or two points of interest are noticeable. Firstly the values for lysine are higher in both the human and the chicken tumour proteins than they are in the normal tissue proteins.

Now it is quite true, as Kocher has remarked, that the recent work of Osborne and Mendel, and also of McCollum and Davis, has taught us the great importance of lysine for the growing animal, but it is hardly justifiable on these grounds alone to connect the high lysine content of tumours with their rapid growth.

On the one hand the increase of this particular diamino-acid in these cases —or indeed the increase of the diamino-acids generally—may merely represent the much greater activity of the cancer cell, expressed in its increased rate of cell-division and its consequent richness in nuclear substance; whereas on the other hand there is the view, apparently held by some, that one or more of these hexone bases acting as what Kocher calls a "growth-factor' may actually be the cause of cell-proliferation. The latter alternative seems altogether untenable, although at the same time it must be borne in mind that there seems every reason to believe that the growth of tumours is controlled by very much the same factors as influence normal growth.

These factors are many in number, for it is realised now that, in order to be adequate for growth, the diet of a young animal must be satisfactory from several standpoints. Thus, not only must the supply of protein in the diet be ample for the nutritive needs of the animal as regards its nitrogen intake, but it must also be satisfactory as regards its amino-acid make-up; while there is also the group of substances which are classed as "accessory growth hormones" which play such an important part in nutrition, but the nature of which we are at present quite unable to define.

All these factors, and no doubt many others, probably influence tumour growth to a greater or less extent, but it seems unwise to infer that any one of them, merely because it occurs in malignant growths to a greater extent than in normal tissues, is the underlying cause of an abnormal rate of cell-division.

Although the average results for all the tumours gives a higher figure for lysine than in the case of the normal tissues, this is not so when the analyses of the normal and pathological breast tissues are surveyed, and again no greatly increased amount of this amino-acid is present in the rapidly growing tumours as compared with the more slowly growing ones.

Of the other amino-acids, high figures for histidine will be observed in some cases, although it is realised that the doubtful accuracy of the arginine determinations has had the effect of yielding higher values for histidine than actually exist, in nearly every case.

Hopkins [1916] has recently demonstrated that this latter amino-acid, together with arginine, is to be regarded as of as much importance to the growing animal as is lysine. His studies have shown that either one of these two compounds can act as the precursor of the purines synthesised in the growing organism, so that they must both be of the greatest importance in the elaboration of nuclear substance.

We may on these grounds designate either histidine or arginine "growth-factors" with quite as much justice as Kocher has done in the case of lysine, although there is little evidence that they are markedly increased in cases of cancer.

The high figures for histidine, to which reference has been made, probably have their origin in at least two facts; firstly, there is a large amount of nucleo-protein in the product analysed, and secondly there is the presence of a certain amount of blood proteins in most of the tumours.

The Rous chicken sarcoma closely resembles the true rapidly growing sarcomata in being exceedingly vascular in character, so that all the growths examined contained a considerable amount of blood. Particularly in the case of the more rapidly growing examples was this so, for it was usual to find these tumours necrotic in the centre, and the cavity filled with extravasated blood and a thick mucoid fluid.

It is therefore conceivable that the high histidine figures may be partly due to the inclusion of a greater or less proportion of blood in the analysis.

Some evidence in favour of this explanation is given by the analysis of the spleen where a similarly high value for this particular determination is recorded.

Returning to a general survey of the results as a whole, it will at once be admitted that the conclusions put forward by Kocher have received no confirmation from this research. Seeking for an explanation of this, we find it in the reliance which this worker has placed on the analysis of normal tissues made by other workers.

Kocher's analyses of tumour proteins are in very good agreement with the analytical data recorded in this communication, as well as with those of Bergell [1907], so that it seems fair to assume that the analytical results for normal tissues which he quotes are inaccurate.

Below are tabulated as far as possible the available analytical figures given by various workers for some normal tissue proteins (Table X). It will be seen that the majority of the figures are in agreement in recording a higher percentage of diamino-acids in these tissues than is given by Wakeman.

In conclusion, it can be said that this research has yielded many results which support the view of Wells [1914], who holds that the cancer cell is very similar, if not identical, in chemical composition with the normal cell of the same type.

All the evidence points to the conclusion that the higher percentage of

diamino-acids which is often present in the proteins of malignant growths is due to the greater proportion of nuclear material which they contain, for similarly high figures for this fraction of the protein molecule are found in the analysis of those normal tissues which possess cells rich in nuclein, as for example, normal human pancreas and spleen.

A similar explanation is applicable to the high diamino-acid content of spermatozoa, which is quoted by Kocher as an example of a germinating tissue rich in hexone bases.

TABLE X.

Analyses of Proteins of Normal Tissues. (*Percentage of Total N.*)

Tissue	Amide N	Humin N	Mon-amino N	Arginine N	Histidine N	Lysine N	Total di-amino N	Observer
Muscle, chicken ...	7·45	2·73	59·85	—	—	—	30·00	Osborne and Heyl [1908]
Muscle, ox	5·50	2·58	64·40	—	—	—	27·23	Osborne and Jones [1909]
Muscle, ox	—	—	—	7·50	1·80	7·60	16·90	Koelker and Slemons, quoted by Kocher [1915]
Muscle, chicken ...	6·63	1·76	61·55	11·10	8·45	9·81	30·27	Drummond
Muscle, human ...	6·30	2·30	62·25	11·20	4·26	12·65	29·90	,,
Liver, human ...	—	—	—	6·24	1·88	6·07	14·19	Wakeman [1908]
Liver, human ...	6·12	3·32	58·20	10·50	8·20	11·76	30·05	Drummond
Liver, chicken ...	8·18	5·53	59·47	9·30	7·30	9·30	27·60	,,
Liver, dog	—	—	—	8·32	2·31	4·81	15·44	Wakeman
Kidney, dog ...	—	—	—	4·19	2·51	6·05	12·75	,,
Kidney, human ...	5·05	3·24	64·40	11·95	5·89	7·00	25·85	Drummond
Haemoglobin ...	5·24	3·60	59·90	7·70	12·70	10·90	31·30	van Slyke [1911]
Serum protein, ox ...	7·05	1·60	58·80	11·05	4·40	13·55	31·75	Hartley [1914]
Serum protein, horse	7·25	1·60	61·95	9·50	5·60	12·80	30·00	,,

Summary.

1. The distribution of nitrogen in a large number of the proteins of human and chicken tumours and normal tissues has been examined. A higher proportion of the diamino-acids generally exists in the case of the tumour proteins.

2. It has been shown that in all probability the diamino-acid content of a tissue varies directly with the amount of nuclear material present.

3. The more rapidly growing and cellular tumours therefore show a higher percentage of these substances than do the slowly growing ones.

4. There is no evidence from the analytical data recorded for the proteins of malignant tumours, which supports the view that the hexone bases are in any way responsible for an increased rate of cell-division.

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XXXIX. THE INFLUENCE OF FLESH-FEEDING ON URINARY CREATININE.

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(*Received August 17th, 1916.*)

Previous Work on the Conversion of Creatine to Creatinine.

In recent years a considerable amount of work has been done to ascertain whether either a conversion of exogenous creatine to creatinine or the converse takes place within the body.

It is generally agreed that creatinine given by the mouth or injected hypodermically is excreted as such to, at least, 80 %, and that there are no indications of its conversion to creatine [Myers and Fine, 1913, 1915; van Hoogenhuyze and Verploegh, 1905].

The question of the conversion of creatine to creatinine however, must still be considered unsettled. Czernecki [1905], Folin [1906], Klercker [cited by Folin, 1906], Lefmann [1908], and Folin and Denis [1914] found no evidence of a conversion. On the other hand, Pekelharing and van Hoogenhuyze [1910], Myers and Fine [1913, 1915], Towles and Voegtlin [1912], and van Hoogenhuyze and Verploegh [1905] found evidence of a conversion, which, however, is small in amount, being in most cases under 5 %, the rest of the creatine being either excreted unchanged or remaining unaccounted for.

Some workers (*e.g.* Levene and Kristeller [1909], McCrudden [1912] and Taylor [1915]) have published results of experiments in which importance is attached to variations in the excretion of creatinine while the subjects of the experiments were upon a flesh-containing diet.

These have either ignored the possible influence of meat-eating on the creatinine excretion, or have assumed, from the work of Folin [1905], that creatinine is of purely endogenous origin, and that its regular excretion is quite unaffected by the ingestion of creatine in any form.

Work done on rabbits is of doubtful value and need not be considered. The daily fluctuations of the excreted creatinine due to the impossibility of obtaining an exact twenty-four hour's secretion of urine, are too great in view of the small amount of conversion which is claimed to be obtained.

Only experiments, in which creatine was given to a subject on a creatine-free diet are worthy of attention.

The most extensive research on this subject is probably that of van Hoogenhuyze and Verploegh [1905], who, in a series of twelve experiments, took each 2 g. of creatine by the mouth, and concluded that creatinine appeared in the urine in small quantity.

An analysis of these results does not seem to bear out this conclusion. One of the six experiments on Verploegh is omitted as it is admittedly in error; and one on van Hoogenhuyze is omitted because a subsequent rise in the creatine excretion, not connected with the experiment, makes comparison impossible.

From 14th September to 3rd October the average creatinine excretion of Verploegh, excluding the days on which the creatine was taken, was 1·917 g. The average of the three creatine days and the following days was 1·905, a decrease of 0·012 g. For van Hoogenhuyze, the figures for the same period were—non-creatine days 1·886 g.; creatine and subsequent days 1·881 g., a decrease of 0·005 g. From the 25th July to 15th February Verploegh's average excretion, excluding creatine days, was 1·996 g. On the two creatine and following days the average was 1·988 g., a decrease of 0·008 g. From 25th January to 9th February van Hoogenhuyze's figures were—non-creatine days 1·886 g.; creatine and subsequent days 1·881 g., a decrease of 0·005 g.

From 25th January to 9th February van Hoogenhuyze's figures were —non-creatine days an average of 1·842 g.; creatine and following days 1·871 g., an increase of 0·029 g.

In view of the amount of creatine taken—2 g.—and of the daily fluctuation in the excretion, the results are not such as would lead to the conclusion that the conversion of creatine to creatinine occurs in the body.

These authors, and the others who conclude that creatinine is a derivative of creatine appear to have been unduly influenced by the work of Gottlieb and Stangassinger [1907, 1908], who believed that they had found in the liver, and to a lesser degree in other organs, an enzyme which converted creatine to creatinine. Valid objections to the acceptance of their results were raised by Mellanby [1908] and by Twort and Mellanby [1913], who showed

that the chemical conditions and presence of bacteria were sufficient to account for the changes observed, and who, under strictly aseptic conditions, were unable to confirm their results. Dakin [1907] could find in the extract of liver, kidney, or duodenal mucous membrane no ferment acting upon creatine. It is interesting that Rothmann [1908] who repeated the work of Gottlieb and Stangassinger, found on one occasion almost twice as much creatinine as could be accounted for by the creatine added.

In any case, the utmost caution should be exercised in drawing far-reaching conclusions from such autolytic experiments, as we do not know that the chemical changes, which occur in an isolated organ in the process of dissolution, are strictly analogous to those that take place in the living body, where the chemical action may be influenced by the activities of other organs of the body.

The influence which an alleged creatinase exerts in converting creatine to creatinine within the body may be disregarded until more certain proofs of its existence are forthcoming.

Though the evidence that creatinine is derived from creatine is wanting, it appears likely that an intimate connection exists between these bodies. This view is indicated by their close resemblance in chemical composition; their mutual connection with the metabolism of muscle [Spriggs, 1907; Shaffer, 1908; Brown and Cathcart, 1909; Pekelharing, 1911]; and by the fact that in the urine of the bird creatine replaces the creatinine found in the urine of mammals [Paton, 1910, 1, 2; Paton and Mackie, 1912].

Experimental.

At the suggestion of Professor Noël Paton, this research was undertaken to ascertain to what extent, if any, the excretion of creatinine is affected by the eating of flesh, and to obtain further evidence of the conversion or non-conversion of ingested creatine to creatinine.

Some of the previous workers used in their experiments flesh, which contains from 3 to 4 % of creatine, while others used pure creatine. In the present series of experiments the influence of flesh and of creatine were investigated separately.

The subjects of the experiment, normal healthy males in the third or fourth decade of life, were put upon a creatine-free diet for several days. Then, on a given day, a weighed amount of flesh or of creatine was added to the diet, after which the creatine-free diet was continued.

Creatine and creatinine were estimated by Folin's colorimetric method, and the total nitrogen by Kjeldahl's method.

The results are recorded below:

A. *The influence of Flesh Feeding.*

TABLE I.

	Amount of urine cc.	Total nitrogen g.	Creatinine g.	Creatine g.	Remarks
Exp. I.	1840	13·22	1·41	—	Creatine-free diet
D. B.	1690	10·53	1·40	—	Creatine-free diet
	1897	15·13	1·50	0·06	227 g. uncooked meat in diet (*i.e.* 0·68 g. creatine)
	1300	11·88	1·43	0·01	Creatine-free diet
	1500	11·55	1·41	—	Creatine-free diet
Exp. II.	1510	11·74	1·63	—	
D.B.	1545	12·32	1·61	—	
	1150	13·17	1·65	0·1	114 g. uncooked meat in diet (0·34 g. creatine)
	1495	13·19	1·64	0·018	
	2280	11·91	1·61	0·02	
Exp. III	770	13·1	1·45	0·04	Creatine-free diet
M. C.	645	12·4	1·48	0·167	227 g. meat cooked
	850	12·2	1·40	0·200	Creatine-free diet
	700	11·5	1·35	—	Creatine-free diet
Exp. IV	1150	12·6	1·6	—	Creatine-free diet
N. M.	900	11·7	1·62	—	Creatine-free diet
	1100	12·9	1·86	0·001	227 g. meat (cooked)
	1100	12·9	1·64	0·002	Creatine-free diet
	900	11·7	1·46	—	Creatine-free diet

In the foregoing experiments, while the diet was creatine-free, except on the days when meat was given, it was only approximately fixed in amount and in composition.

In the two following experiments a fixed diet of known composition was maintained. In Exp. V, it consisted of oatmeal, pease-meal, white bread, dried skimmed milk, sugar, butter, and cocoa, and contained, approximately, protein 63 g., fat 80 g., and carbohydrate 320 g.

It was continued for six days. On the seventh day 125 g. of meat were added to the diet. An analysis of 125 g. of the same flesh was made by the method described by Brown and Cathcart [1909]. There was found 0·38 g. of creatine and no creatinine. The meat was eaten raw to avoid the possibility of a conversion to creatinine taking place in the cooking. Observations

made by Noël Paton show this to occur to a small and varying extent[1]. In Exp. VI the pease-meal of the diet was replaced by bread. Table II gives results of the urinary analysis.

TABLE II.

	Amount of urine cc.	Total nitrogen g.	Creatinine g.	Creatine g.	Remarks
Exp. V	1017	10·35	1·46	—	Fixed creatine-free diet
J. B. O.	1360	10·46	1·52	—	Fixed creatine-free diet
	1400	10·08	1·44	—	Fixed creatine-free diet
	960	12·52	1·62	0·05	125 g. uncooked meat extra (0·38 g. creatine)
	1184	13·60	1·47	—	Fixed creatine-free diet
	757	10·96	1·32	—	Fixed creatine-free diet
Exp. VI	1000	8·90	1·70	—	Fixed creatine-free diet
J B. O.	710	9·72	1·77	—	Fixed creatine-free diet
	680	9·99	1·85	—	170 g. uncooked meat added to diet (0·5 g. creatine)
	685	9·61	1·60	—	Creatine-free diet

B. *The ingestion of creatine.*

One experiment on the ingestion of pure creatine was made.

The diet consisted of the same ingredients as in Exp. V, and contained approximately of protein 60 g., fat 79 g. and carbohydrate 315 g. It was continued for ten days. On the third, sixth and ninth days 0·5 g. of creatine was taken dissolved in water. The creatine used was a preparation of Merck's.

(It was analysed and found to contain a trace of creatinine.)

Table III gives the record of the urinary analysis.

[1] We are indebted to Prof. Noël Paton for the following figures which indicate the varying extent to which cooking causes the conversion of creatine to creatinine in flesh.

Material	Total creatinine %	Creatinine %	Creatine %
Salt beef cooked	0·234	0·109	0·125
Horse-flesh cooked at knacker's	0·244	0·047	0·197
Mince cooked at home ...	0·260	0·096	0·164
Steak cooked in laboratory			
[Raw]	0·346	0·0194	0·3266
Grilled	—	0·0262	0·3198
Stewed	—	0·0210	0·3250
Boiled	—	0·0217	0·3243
Steak	0·315	0·0214	0·2936
Valentine's meat juice	0·696	0·338	0·358

TABLE III.

	Amount of urine cc.	Total nitrogen g.	Creatinine g.	Creatine g.	Remarks
Exp. VII	980	8·74	1·78	—	
J. B. O.	670	8·71	1·8	—	
	640	7·96	1·8	—	
	890	8·68	1·77	0·03	0·5 g. creatine
	760	8·71	1·8	—	
	730	8·71	1·808	—	0·5 g. creatine
	660	8·46	1·8	—	
	790	10·46	1·8	0·2	0·5 g. creatine

Discussion of Results.

A. *Flesh.* In every instance, there follows on the ingestion of the meat an increased excretion of creatinine, which is confined chiefly to the day on which the meat is eaten. The following table summarises the series, showing the average in the preliminary days of creatine-free diet and the amount excreted on the day on which the meat was taken.

TABLE IV.

Creatinine in grams.

Exp.	Subject	Preliminary period average	Day of meat ingestion	Increase	Amount of meat ingested
I	D. B.	1·405	1·5	0·095	227 g. raw
II	D. B.	1·62	1·65	0·03	114 g. raw
III	M. C.	1·43	1·48	0·05	227 g. cooked
IV	N. M.	1·61	1·86	0·25	227 g. cooked
V	J. B. O.	1·47	1·62	0·15	125 g. raw
VI	J. B. O.	1·735	1·85	0·115	170 g. raw

It is seen that there is no parallelism between the amount of flesh eaten and the increase in the excretion of creatinine. The results show that estimations of the excretion of creatinine, while a subject is upon a flesh-containing diet, are quite devoid of value, even though the amount of flesh in the diet be kept constant.

B. *Creatine.* Of the occasions on which pure creatine was given, the first shows a decrease in the output of creatinine of 0·03 g.; the second a slight increase of 0·008 g., and the third, no change. These small variations are within the limits of the normal fluctuation. This experiment appears to afford no evidence that ingested creatine is converted within the body to creatinine.

Why, then, is there an increased creatinine output after a flesh diet, and

why does not a similar increase take place after the ingestion of pure creatine? There are at least, three possible explanations:

(*a*) Creatine (or creatinine) may be "protected" when administered in flesh from destruction in the alimentary canal, and so a greater absorption of these bases may take place. Twort and Mellanby [1913, p. 45] have shown that flesh may "protect" added creatine from destruction (*in vitro*) by certain creatine-destroying bacteria of the intestinal canal. They suggest that this bacterial action is the cause of the loss of creatine and the retention of creatine nitrogen when pure creatine is given by the mouth to a subject on a creatine-creatinine-free diet with a moderate or low nitrogen intake. Against this must be put the finding of MacNeal and Chace [1913], viz. that, under normal conditions, the upper portion of the gut is comparatively sterile. Myers and Fine [1915] urge that, in any case, the loss of creatine by bacterial action, would be inappreciable, because of the rapid rate of absorption.

(*b*) In addition to the preformed extractable creatine, another precursor of creatinine may exist in flesh which after absorption, at a later stage of metabolism, may yield creatinine. One of us [Burns, 1916, 1] has shown that in the developing chick guanidine is formed in muscle, in point of time, before creatine and reaches its highest concentration at the point when creatine appears in detectable amounts. Further, the administration of the salts of guanidine [Burns, 1916, 2] may produce a small increased daily output of creatinine per kilo. body weight. Flesh contains not only creatine (or some bodies which post-mortem give rise immediately to creatine and more slowly to creatinine) but free guanidine, methyl-guanidines and complexes which are readily oxidised to guanidine. It might well be that under different conditions of oxidation, *e.g.* at different stages of metabolism these various catabolites might be produced [Ditman and Welker, 1909].

(*c*) The increased creatinine excretion after the ingestion of flesh may be due to the specific dynamic action of the various products of flesh catabolism. It is well known that any factor which increases the heat production of an animal increases the creatinine output, *e.g.* fevers [Leathes, 1907] and artificial hyperthermia [Myers and Volovic, 1913].

The increased creatinine output got by Myers and Fine [1915] after the ingestion of pure creatine may possibly be explained as the result of the diuresis. Burns and Orr [1914] pointed out that the production of a diuresis by the ingestion of water markedly and immediately raised the creatinine coefficient. The administration of 1 g. of creatine (in 250 cc. of warm water) to Myers increased the volume of urine from 570 to 910 cc. and produced no increase

in the creatinine or creatine of the urine. The addition of 2 g. of creatine raised the urine volume to 1330 cc. and its creatinine content by 0·03 g. The same amounts of creatine taken by Fine resulted in urinary alterations of water 645 cc. to 1100 and 1205 cc.; of creatinine 1·29 to 1·26 and 1·32 g. (20 and 29 % of the ingested creatine was eliminated as such in this case.)

Burns and Orr [1914] obtained similar increases in the creatinine output on the ingestion of water.

		Creatinine g.		Creatine g.	
Exp.	Amount of water	Before	After	Before	After
III.	3 litres	1·31	1·33	—	—
VII.	,,	1·33	1·36	0·01	0·01

Summary.

1. The ingestion of flesh causes an increased excretion of creatinine and generally the appearance of creatine in the urine.

2. The amount of the increase is not proportional to the amount of flesh taken.

3. Although cooking increases, to a variable extent, the amount of creatinine present free in flesh, cooked meat does not necessarily cause a greater increase in urinary creatinine than uncooked flesh.

4. Pure creatine administered per os is only to a slight extent eliminated as such and does not markedly alter the creatinine excretion.

The above experiments were carried out by the aid of a grant to one of us (D. B.) from the Carnegie Trustees to whom we desire to express our indebtedness. Our thanks are due to Prof. Noël Paton for suggesting this research and to him and to Prof. Cathcart for kindly criticism while the experiments were in progress.

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XL. METHODS FOR THE ESTIMATION OF MIXTURES OF FOUR OR MORE CARBOHYDRATES, INVOLVING OXIDATION WITH BROMINE.

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(*Received September 4th, 1916.*)

In the course of investigations upon the diurnal changes in the carbohydrate content of green plant tissues, many analytical difficulties were encountered, and it was therefore decided critically to examine existing methods and to devise others if possible. The work was unavoidably delayed and since there is but little prospect of bringing it to completion in the near future, we have decided to publish what has been outlined up to the present. While carrying out this work a series of papers has appeared by Messrs Davis and Daish [1913] and their collaborators, dealing with the same subject and we have been much indebted to them.

The Carbohydrates of the Green Leaf.

The five carbohydrates usually found in foliage leaves are starch, sucrose, *d*-glucose, *d*-fructose and maltose, and in addition to these sometimes small quantities of pentoses are known to be present. The estimation of starch has been fully discussed by Davis and Daish [1914] and that of the pentoses by Davis and Sawyer [1914] and by Daish [1914]. In the present work, therefore, only the remaining four sugars will be considered.

Reduction Method.

Before beginning this work, it was necessary to find a suitable method of quantitatively estimating reducing sugars gravimetrically. As all methods based on the use of Fehling's solution are liable to error, owing to the solvent action of the hot sodium hydroxide on cuprous oxide, it was decided

after careful tests to use Kendall's method [1912], weighing the copper as cupric oxide as advocated by Davis and Daish [1913] and by Zerban and Naquin [1908], the action of reducing gases being avoided by heating the Gooch crucible containing the precipitate inside a larger porcelain or nickel crucible.

It has been stated by some that cupric oxide is hygroscopic and that this constitutes an objection to the method. However we have found no trace of this hygroscopic property throughout the numerous experiments carried out during this research, provided the carbonate used was pure. We have noticed, however, that if there is any iron present as an impurity in the carbonate used for reductions, then hygroscopic properties are observed, apparently in the cupric oxide, in reality due to the presence of red oxide of iron (Fe_2O_3), and moreover in such cases, we observed that the cuprous oxide was precipitated yellow whereas in all other cases it came down red. The blue amphibole asbestos for use in the Gooch crucibles must also be treated as insisted on by Davis and Daish, in order to ensure its constancy in weight when exposed to hot alkaline solutions.

Kendall's procedure, very briefly described, consists in adding to the sugar solution which has been made up to 100 cc. with distilled water, solid salicylic acid, a solution of copper sulphate and a strong solution of potassium carbonate in the order named[1]; the mixture having been gently shaken is heated in a rapidly boiling water-bath for 20 minutes. Boiling should begin again within from one to two minutes of the introduction into the bath of the cold solution. Kendall has prepared tables for the reducing powers of glucose, invert sugar, maltose and lactose, but since a table for fructose was not included, one has been prepared by us and used for our analyses [1916].

Analysis of Sucrose, Maltose, *D*-Glucose and *D*-Fructose.

(1) *Sucrose.*

The estimation of sucrose is usually carried out by inversion under Clerget conditions or by some appropriate modification of the method. When, however, maltose is present treatment with a mineral acid results in hydrolysis of a portion of this sugar. To avoid this citric acid in from 2 % to 10 % solution has been employed. Davis and Daish [1913] have studied this point carefully and recommend the use of invertase in preference, or at least

[1] The amounts employed are: salicylic acid 5 g., copper sulphate 15 cc. of a solution containing 133·33 g. of pure crystals per litre, and potassium carbonate 25 cc. of a solution containing 600 g. of anhydrous salt per litre.

as a check upon, hydrolysis with citric acid. The invertase may most conveniently be prepared and preserved according to Hudson's [1914] directions.

The calculation of the amount of sucrose is based upon the readings of the polarimeter before and after inversion or upon the copper reducing power of the solution. The changes in these two values should of course give concordant results; but sucrose itself has a slight reducing power when mixed with reducing sugars, so that a slight discrepancy will usually be observed.

When the amount of sucrose has been determined the rotation due to it may be calculated and hence the rotation of the remaining three reducing sugars in the mixture. One other sugar has now to be determined directly (as a solution of equations containing three unknowns is not satisfactory). The most usual sugar to determine now is maltose, and then by subtracting the rotation due to sucrose and maltose from the initial observed rotation and deducting the amount of copper due to maltose from the observed copper reducing power of the original solution, there remain two equations for the two unknowns, glucose and fructose, which may easily be found.

Now maltose may be estimated either by Brown and Morris's method [1893], of hydrolysis with hydrochloric acid under standard conditions, or by Davis and Daish's method [1913] of fermentation with pure cultures of maltase-free yeasts, which seems to have given very accurate results in the hands of its originators. But as each estimation requires at least twenty-one days and involves the use of a pure culture of a particular yeast it must be conceded that it is cumbersome. Accordingly a method is here suggested whereby fructose and not maltose is determined directly in the remaining mixture of three sugars. This method is outlined in the following pages.

(2) *Oxidation methods in acid or neutral solution.*

In all cases in which it is desired to estimate the separate sugars in a mixture of glucose, fructose and maltose, it is necessary as previously pointed out either to have three equations or to have a means of estimating one sugar by some special method.

At the outset it seemed that use might be made of the differential action of bromine upon the ketonic and aldehydic sugars. The former are scarcely oxidised at all, whereas the latter readily yield aldonic acids.

Bunzel [1909] has carefully studied the mechanism of the oxidation of glucose by bromine. He found that the amounts of acid and of bromide produced, as well as the amounts of glucose and of bromine disappearing, are

equal to those calculated on the hypothesis that one molecule of bromine oxidises one positive glucose ion to gluconic acid. Thus when acidified with hydrochloric acid, 0·1 normal, the equation is as follows:

$$C_6H_{13}O_6^+ + Cl^- + Br_2 + HOH = C_6H_{12}O_7 + HCl + 2HBr.$$

The correctness of this view is supported by the constancy of the velocity constant, K, in the following equation, even under widely varying conditions of acidity and with different concentrations of bromine and glucose·

$$\frac{dx}{dt} = K \cdot C_{C_6H_{13}O_6^+} \times C_{OH^-} \times C_{Br_2\,(free)}.$$

From this it is seen that the rate depends upon three factors: (*a*) the concentration of the positive glucose ion $C_6H_{13}O_6^+$; (*b*) the concentration of the hydroxyl ions; (*c*) the concentration of the free bromine in solution.

Bunzel has shown that provided the concentration of the hydrogen ions is over 0·1 normal the hydroxyl ion factor may be entirely neglected. The velocity under these conditions is proportional to two variables, the concentration of the glucose and of the free bromine. Bunzel has also proved that in an acid medium the positive glucose ion goes quantitatively into gluconic acid. This may be expected to give rise to its lactone which is further oxidised by bromine very slowly.

The oxidation of the negative glucose ion $C_6H_{11}O_6^-$ probably yields a large number of acids. These have been studied by Nef [1907, 1910, 1914]. Since glucose is a very weak acid its dissociation in this manner is almost completely prevented by strong acids, with which it forms oxonium salts and ionises to $C_6H_{13}O_6^+$ as previously mentioned.

From these considerations it is evident that in 0·1 normal acid saturated with bromine and maintained in this condition, the rate of oxidation of glucose will be that of a unimolecular reaction. For the other variable, the concentration of bromine has now been reduced to a constant. This being so the same fraction of the glucose present will be oxidised to gluconic acid in a given time, irrespective of the initial concentration, provided the temperature is maintained constant.

The reaction under these conditions is a perfectly definite one and should be available for the estimation of glucose. The standard conditions will be discussed again in relation to the actual procedure adopted.

The action of bromine upon maltose in the cold is quite similar to that upon glucose, maltobionic acid being the product. Fructose is, however, usually considered to be almost unaffected by this halogen. At the outset

it appeared possible to remove the glucose and maltose quantitatively by oxidation with bromine. The reducing power of the solution would then have been due to fructose alone. Direct experiment, however, has shown that this is not rigorously exact, for when sufficient time has elapsed for the oxidation of all the maltose a small proportion of the fructose has in some way been destroyed. Moreover the oxidation of the glucose is never complete, or to be more accurate, prolonged treatment with bromine always leaves a solution which has still some reducing action upon Kendall's solution, corresponding to about 2 % of the glucose originally present. With maltose too the residual reducing power after treatment with bromine, though negligible, is quite perceptible.

This residual reducing power of glucose solutions most probably has its origin in the formation of gluconic lactone from the first formed gluconic acid as its concentration increases. That this is the case is borne out by the fact that all our attempts to isolate the phenylosazone of glucose from such solutions were unsuccessful, giving rise only to the production of gluconic phenylhydrazide. Moreover two experiments performed under the standard conditions and at average temperatures differing by about two degrees showed that oxidation by bromine for 66 hours afforded a solution with a larger residual reducing power than a similarly conducted oxidation for 42 hours. This apparently indicates the formation of the lactone on prolonged oxidation. A similar explanation is most likely applicable to the very slight residual reducing power observed also in maltose solutions.

Details of these analyses are given in the experimental portion of the paper.

The action of bromine upon these three sugars was studied at temperatures between 0° and 35° before all hope of a very exact separation was abandoned.

Since, however, the action may be reduced to the monomolecular type by maintaining the bromine in saturated solution, and by having a suitable excess of mineral acid, it is obvious that constants can be determined for each sugar, representing the fraction of its initial reducing power remaining after treatment with bromine under standard conditions. Accordingly such constants were found for experiments carried out at room temperature, after an attempt to work at 0°; the latter steady temperature was not suitable since the solution became of indefinite composition owing to the separation of very large amounts of bromine hydrate. The solutions were shaken continuously to ensure complete saturation with bromine throughout the period of oxidation. Shaking was effected by rotating the bottles, which

were partly filled with glass beads, about a horizontal axis. To ensure a more uniform temperature they were placed in a tank filled with water, and covered to exclude the light. A small electric motor running at about three thousand revolutions a minute was geared down suitably to rotate the solutions.

The equations available for determining the composition of a mixture of three sugars are then those usually employed for polarisation and reduction, with a third similar to the reduction equation but introducing a constant for residual sugar after halogen oxidation, thus:

$$(1) \quad 100\,[\alpha]_D^{20} = x\,[\alpha]_{D_g}^{20} + y\,[\alpha]_{D_f}^{20} + z\,[\alpha]_{D_m}^{20},$$

where x, y and z represent the amounts of glucose, fructose and maltose respectively.

$$(2) \quad w_1 = k_g x + k_f y + k_m z,$$

where k_g represents the weight of cuprous oxide obtainable from Kendall's solution by 100 mg. or other suitable quantity of glucose; k_f and k_m have similar meanings.

$$(3) \quad w_2 = k_g . a . x + k_f . b . y + k_m . c . z,$$

where a, b and c are the percentages of residual sugar after treatment with bromine under the standard conditions.

On substituting the experimental data in the above equations an attempt was made to solve for the three unknowns; but in such methods of solution experimental errors are often largely multiplied and the magnitude of the errors so introduced in the mathematical operations was found in this case to be so great as to render the method useless.

Now, when the time allowed to elapse was 66 hours at room temperature (17°–18°) the values for the residual sugar constants were $a = 0{\cdot}033$; $b = 0{\cdot}970$; $c = 0{\cdot}006$. It will be noticed that the sum of these constants is 1·009, from which it follows that it is very nearly correct to assume that no fructose has disappeared, but that the other two sugars have been completely oxidised away.

It has been found that part at least of the small loss of fructose is due not to its oxidation by bromine but to its decomposition by the decinormal acid added to the solution, and further reinforced in the case of mixtures of the three sugars by the hydrobromic acid generated in the course of the reaction. Experiments proving this are shown in the experimental portion of this paper.

In order to test by direct experiment the supposition that under certain

standard conditions it is legitimate to assume that all the residual reducing power of the solution is due to fructose when glucose and maltose have also been present at the start, solutions containing these three reducing sugars were made up. They were then examined by the polarimeter, and the reducing power both before and after oxidation with bromine was determined for suitably diluted aliquot portions of the original solutions by Kendall's method[1]. The results are shown in Table I. It must be remembered that, of the three, fructose is the only sugar which has been directly determined. The quantities of the other two had to be found by solving the simultaneous equations (1) and (2) for two unknowns. The multiplication of errors is not nearly so serious as with three unknown quantities, yet the accuracy to be expected is not of the same degree as in direct determinations.

TABLE I.

	I		II		III	
Temperature	13°–16°		17°–18°		10°–11°	
Time of oxidation	42 hours		66 hours		66 hours	
	Taken g.	Found g.	Taken g.	Found g.	Taken g.	Found g.
Fructose	0·7686	0·7650	0·4166	0·4150	0·4975	0·4615
Glucose	1·0260	0·9904	0·6955	0·5886	0·4910	0·4556
Maltose	0·8950	0·9863	0·6615	0·6953	0·4888	0·4778
Total	2·6896	2·7417	1·7736	1·6989	1·4773	1·3949

These results indicate that the method is capable of giving fairly accurate values for fructose in the presence of either or both of the other reducing sugars. The details of the estimation may possibly be improved so as to render the analysis of greater accuracy. In the latter quality this method is not so good as that of Davis and Daish but inasmuch as it can be carried out in three days instead of in three weeks, it may be found to commend itself for certain purposes.

(3) *Oxidation methods in alkaline solution*[2].

In alkaline solution the sugars are rapidly oxidised by atmospheric oxygen with the production of a brown colour. The relative rates of oxidation under these conditions are the same as in acetic acid, fructose being the most readily attacked. This difference between the reactions in acid and alkaline solution is most simply accounted for by the hypothesis that the sugars are

[1] The solutions for standard oxidation consisted of 50 cc. sugar solution diluted to 70 cc. and then 10 cc. normal sulphuric acid added and finally bromine 5 cc.

[2] This problem was forced upon our notice through the accidental addition of a slight excess of alkali in neutralising, as explained in the "Experimental" portion of this paper.

weak acids, the ionisation of which is hindered by other acids whereas the sodium salts formed in alkaline solution are readily ionised. This results in the liberation of very unstable and easily oxidisable negatively charged sugar ions. Mathews [1909] noticed that the rate of oxidation of glucose and other sugars in alkaline solution increased as the reaction proceeded. He adduced reasons for the belief that the glucose molecule is broken up into a number of active reducing molecules under these conditions. This process takes place even in the absence of atmospheric oxygen, and so has no connection with any oxidation processes in which gaseous oxygen is concerned, though intra-molecular oxidations are not excluded.

When alkaline solutions of the three reducing sugars glucose, fructose and maltose are exposed to gaseous oxygen they are readily oxidised as has already been mentioned. In consequence one might expect a decrease in their reducing power towards copper solutions. On testing this point directly it was found that the change in reducing power was an increase, followed by a decrease. In one case for example the procedure was as follows: a 2 % solution of each of the three sugars was mixed with an equal volume of normal sodium hydroxide. Before mixing, immediately after mixing and at various intervals of time the reducing powers of the solutions were determined by means of Fehling's solution. In the first case an equal volume of water was added to the sugar solution, in order that the analyses might be exactly comparable with the others of the series. The flasks were allowed to stand at room temperature, about 15°, and were not shaken up with air. At the same time in these preliminary experiments no precautions were taken to avoid oxidation. The results are shown in Table II.

Since there is a definite change in the reducing powers of the sugars when allowed to stand in half-normal sodium hydroxide it is evident that a set of constants can be obtained for the different sugars showing what alteration takes place in each under standard conditions. In obtaining these due regard must be paid to the most suitable times, temperatures and concentrations. Means must also be taken to prevent any appreciable amount of oxidation, for example by filling the flasks with hydrogen. If it so happened that all the reducing sugars were altered in the same proportions the effect would only be to alter the magnitudes of the usual constants for the reducing powers of these substances towards the various alkaline copper solutions in use. But experiment has shown that the change in the reducing powers of the various sugars takes place to very unequal extents, as after an initial increase, the rate of decrease is most rapid for fructose.

The results of these experiments are tabulated below; the concentrations recorded refer in each case to the mixture not to the original strengths of the separate solutions.

TABLE II.

Action of N/2 sodium hydroxide on sugars at room temperature.

Glucose 1 %			Glucose 3 %			Fructose 1 %			Maltose 1 %		
Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power
0	0	100·0	0	0	100·0	0	0	100·0	0	0	100·0
0	15	132·0	0	7	103·5	0	8	132·3	0	7	137·4
0	25	131·5	0	15	104·7	3	37	132·3	3	35	128·8
0	44	131·5	0	28	104·7	23	45	83·3	23	0	115·3
21	55	95·7	4	25	102·3	47	0	66·4	46	45	108·9
22	8	94·6	24	20	94·6	—		—	—		—
43	40	88·0	—		—	—		—	—		—

TABLE III.

Action of 2N sodium hydroxide on sugars at room temperature.

Glucose 1 %			Glucose 2 %		
Time hrs.	mins.	Reducing power	Time hrs.	mins.	Reducing power
0	0	100	0	0	100
0	5	113·2	0	7	104·4
4	50	117·0	5	47	151·1
24	20	88·5	23	50	95·3
126	30	34·0	—		—

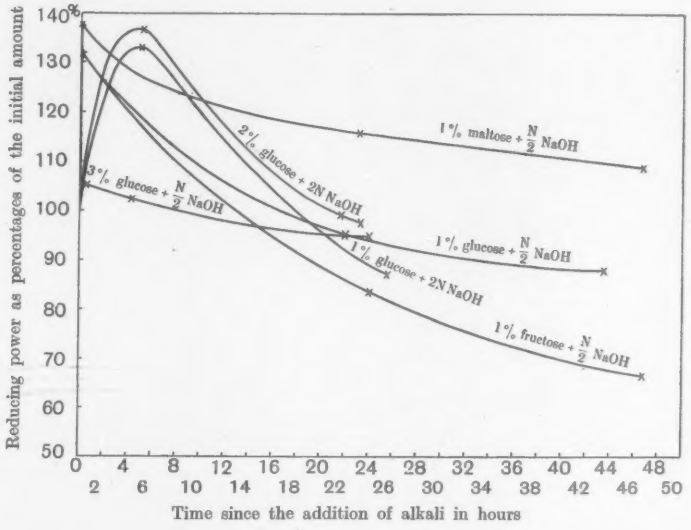
The results are graphically shown in the figure.

Considering the N/2 alkali, with the 1 % solutions there is a very rapid rise in reducing power consequent upon the addition of alkali. This rise is identical in magnitude for glucose and for fructose within the limits of accuracy of the method. With maltose a somewhat greater rise is found. It must however be pointed out that the hexoses are present in equimolecular proportions, viz. 10/180 normal (= 0·055 N), whereas the molecular concentration of the maltose is far smaller, 10/342 normal (= 0·029 N).

With 3 % glucose the increase in reducing power is far smaller, and attains its maximum value more slowly.

Mathews [1909] clearly showed that there was an increase in the number of active reducing molecules consequent upon the addition of alkali. This he ascertained by measuring the rate of absorption of oxygen by a glucose

solution which was kept over mercury after being made alkaline. From the preceding tables it is evident that the increase is very considerable and a maximum is quickly reached. However the relationship between the quantity of alkali added and the percentage of sugar undergoing change requires further investigation. It has recently been found by Powell [1915] that sodium hydroxide reacts with glucose and fructose with the formation of sodium salts, each molecule of sugar taking up one sodium ion. This conclusion was deduced from the study of the retardation in the hydrolysis of ethyl acetate by the hydroxide, which follows the addition of a sugar.


Reducing power as percentages of the initial amount
140%
130
120
110
100
90
80
70
60
50
0 4 8 12 16 20 24 28 32 36 40 44 48
2 6 10 14 18 22 26 30 34 38 42 46 50
Time since the addition of alkali in hours
1 % maltose + N/2 NaOH
2 % glucose + 2N NaOH
3 % glucose + N/2 NaOH
1 % glucose + N/2 NaOH
1 % glucose + 2N NaOH
1 % fructose + N/2 NaOH

Before becoming acquainted with Powell's results an attempt was made by us to obtain evidence concerning the union of sugar and alkali by means of conductivity measurements. The conductivity of 1 % glucose solution was measured—it was found to be about half that of the Dublin water supply —and also the conductivity of seminormal sodium hydroxide. These quantities were chosen as being those employed in obtaining the results shown in Table II, though as the sugar solution is only 0·055 normal it would perhaps have been advisable to use more dilute alkali. Then however a compound, if formed, would be almost completely ionised, whereas with one component in large excess such ionisation would be diminished. The

objection to using a strong sugar solution is that the viscosity changes would then begin to have a serious effect on the conductivities.

The conductivity of a solution composed of alkali and glucose in the same final concentrations was then measured at various intervals in a closed vessel.

TABLE IV.

Solution		Conductivity in mhos at 0°, $\times 10^5$
Glucose 1 % (0·055 N) ...		2·181
Sodium hydroxide 2 % (0·5 N)		5661·0
Glucose 1 % ... ... Sodium hydroxide 2 %	15 minutes after mixing	5272·0
,, ,,	55 minutes ,,	5124·0
,, ,,	105 minutes ,,	5121·0
,, ,,	22·5 hours ,,	5170·0
Sodium hydroxide 2 % taken fresh from the corked flask, after standing in it for 23 hrs.		5214·0

Now neglecting the slight effect that the alteration in viscosity due to the glucose will have upon the conductivity, and assuming that the glucose combines in unimolecular proportions with the hydroxide, a conductivity of $C \times 10^5 = 5095$ should result if the conductivity varied directly as the concentration. This value is in reality slightly too low, for the more dilute solutions have higher molecular conductivities, 0·45 N hydroxide conducting better relatively than does 0·50 N. However the increase in viscosity acts in the opposite direction. Thus a comparison of the observed conductivity after 105 minutes, $C \times 10^5 = 5121$, with the calculated conductivity, $C \times 10^5 = 5095$, points to the formation of a compound between the sugar and alkali. This takes place to the extent of 95 % of the theoretical amount, the compound being dissociated to the extent of 5 %, or taking the final conductivity as $C \times 10^5 = 5214$, the dissociation amounts to 21 %. On the whole however it would be very unwise to conclude that the changes in such an unstable reaction-mixture as an alkaline sugar solution could be accurately represented by any one reaction.

In concluding this section it may be remarked that though the study of the oxidation of the sugars in alkaline solution has not lent itself to any quantitative method for their estimation when two or more are present together, yet it has shown what grave errors may result if sugar solutions are allowed to become alkaline in the course of analyses. Such solutions should always be neutral or faintly acid.

(6) *Oxidation by cupric acetate in presence of acetic acid.*

The use of Barfoed's solution is well known as a qualitative test to distinguish glucose and fructose, which reduce this reagent, from maltose and lactose, which under the specified conditions are without this action. All attempts to use the method for quantitative work have failed, since the disaccharides effect some reduction when the boiling is continued sufficiently long to oxidise all the hexoses. The amount of acetic acid present is also an important factor, and during the heating some of it is driven off.

This reaction has been very carefully studied by McGuigan [1907], Mathews and McGuigan [1907] and by Bunzel [1908]. It was found by Bunzel that the reaction is of the fifth order. However it seemed possible that it might be reduced to the first order, by adopting certain standard conditions, and be then available for the analysis of mixtures by differential oxidation of the sugars. With this object sodium acetate, 10 g., was added to 100 cc. of Barfoed's solution[1] to ensure a constant acidity of small magnitude. A large excess of the reagent was also taken, 100 cc. for 25 cc. of a 2 % solution of glucose, so that the change in the concentration of the copper should not be of too considerable magnitude. Furthermore the boiling of the mixture was effected under a reflux condenser. The cuprous oxide was collected in a Gooch crucible and weighed as cupric oxide. The following table shows that the solvent action of the acetic acid upon the oxide is too great to allow of accurate quantitative estimations by the method. Even the addition of the sodium acetate did not suffice to reduce this to negligible proportions.

TABLE V

Cupric oxide from 100 *cc. Barfoed's solution,* 10 *g. sodium acetate and* 25 *cc. of* 2 % *glucose, the latter being added cold to the former already heated to* 90° *in a water-bath. Solution cooled under water-tap at end of heating.*

Time of heating	Cupric oxide
10 min.	1·1296 g.
15	1·2773
20	1·2257

Furthermore it was found that when 1 % glucose was used, and the heating was continued for fifteen minutes, the yield of cupric oxide was 0·8234 g.; indicating a reducing power nearly half as great again as for a 2 % solution.

[1] Copper acetate crystals 66 g. per litre, glacial acetic acid 10 cc. per litre.

A similar attempt using the mono- and di-hydrogen sodium phosphates instead of sodium acetate was also found to be unsatisfactory. However though this attempt at reaching suitable conditions is a failure it is perhaps worth recording to save others from similar waste of time.

Experimental.

The sugars used in the course of this investigation were the following: (*a*) for preliminary work, the pure anhydrous glucose, fructose (laevulose) and maltose of commerce; (*b*) for accurate work, determinations of constants, etc., Kahlbaum's sugars were used as a rule, the fructose being the "laevulose from inulin I." In some experiments pure commercial glucose and maltose were employed, after recrystallisation from alcohol three times.

The powdered sugars were kept in desiccators over sulphuric acid for long periods. The residual moisture was determined by heating a small portion, about 2 g., in an exhausted flask connected with drying tubes of phosphoric oxide at air temperature which led to a Geryk pump. The temperature at which the sugars were heated was that of the water-oven. When drying fructose it is advisable to use a lower temperature, about 50°, at first if the sample contains any appreciable amount of moisture. The "laevulose from inulin I" however did not melt and remained perfectly white when heated to 98° at atmospheric pressure in a vessel containing phosphoric oxide. Its purity was further tested by determining its specific rotation, in 5 % solution at 17·4° in a 20 cm. tube, using a Schmidt and Haensch polarimeter. When corrected for temperature it gave $[\alpha]^{D}_{20} = -92{\cdot}3°$, which agrees closely with the value chosen for use throughout this work, viz. $-92{\cdot}5°$. Later on a polarimeter by Adam Hilger was employed.

Anhydrous maltose was prepared by distillation with alcohol and benzene as recently described by us [Atkins and Wilson, 1915], but owing to its extremely hygroscopic nature it was deemed advisable to employ the hydrate for quantitative work, and to correct for the small excess of moisture beyond the theoretical amount of water of crystallisation.

Action of Bromine on Glucose, Fructose and Maltose.

To study this action solutions of the sugars were saturated with bromine, and allowed to stand in the dark over excess of the liquid halogen. In some experiments the bottles were shaken from time to time to maintain saturation. In determining constants the shaking was continuous.

At the end of the period chosen the liquid bromine was removed by means of a separating funnel and what remained over was converted into hydrobromic acid by passing in sulphur dioxide till the solution was nearly colourless. The last traces were destroyed by the careful addition of a solution of sulphurous acid from a burette. The mixture was then almost neutralised with solid potassium carbonate, taking care to leave the solution faintly acid at the end. A blank experiment showed that this procedure afforded a solution which had no reducing action on alkaline copper solutions.

The reducing power of the sugar solutions after treatment with bromine was determined by Kendall's method, but the cuprous oxide was oxidised by heating in air, and weighed as cupric oxide. The amounts of fructose were found from a table prepared by the authors in exact accordance with Kendall's method of drawing up tables for the other sugars [1916].

The following tables show the results of the preliminary experiments.

TABLE VI.

Oxidation of glucose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of glucose remaining. Initial concentration = 100 %
Room 13°–16°	10	16 days	2·5
„	10	12 „	3·0
33°	2	4 „	8·9
0°	1½	90½ hours	50
0°	1½	91¾ „	69·13
0°	1½	90 „	34·77 35·76* 53·03† solution shaken
	Same experiment		

TABLE VII.

Oxidation of fructose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of fructose remaining. Initial concentration = 100 %
Room	12½	16 days	97
„	11½	12 „	98
40°–45°	11	4 „	92
22°	5	4 „	95
0°	1	90 hours	100·0* 101·6† solution shaken
	Same experiment		

TABLE VIII.

Oxidation of solution of maltose by bromine in approximately saturated solution.

Temperature	Approximate percentage strength of sugar solution	Time	Percentage of glucose remaining. Initial concentration = 100 %
Room	11	16 days	0·0
„	12	12 „	0·0
33°	1½	4 „	2·3
0°	2	90¼ hours	37·4
0°	1	92 „	50·8
0°	1	90 „	{19·50* 30·75†} solution shaken
	Same experiment		

The figures marked with an * resulted from experiments where the solutions had been allowed to stand for four hours with a slight excess of potassium carbonate. Those marked with a † were given by the same solution after standing for two days. The solid carbonate was added to neutralise, and the slight excess varied in each case. The delay was accidental, only the glucose being estimated immediately after neutralisation in the case of these experiments carried out at 0° during 90 hours.

The action of alkali in bringing about an increase in the reducing power of the sugar solutions was here forced upon our notice for the first time. The phenomenon was studied at some length later on, and has been already mentioned in the earlier portion of this paper.

Inspection of the foregoing results shows that fructose is not appreciably oxidised at 0°, when allowed to stand for nearly four days. Prolonged action at higher temperatures appears to destroy some of this sugar.

It must however be noted that the experiments at 0° were carried out with "laevulose from inulin I," which is quite free from glucose. The others were performed with crystallised laevulose, Kahlbaum's also, and any small quantity of glucose it may have contained would have been oxidised and so cause the loss of fructose to appear too great. We suggest that the purity of samples of laevulose might be tested by allowing their solutions to stand with bromine at air temperature for one day or at 0° for four days. The loss of reducing sugar will then be due almost entirely to the destruction of glucose, maltose or other aldehydic sugar. Our experiments on fructose are in agreement with those of Votoček and Nemecek [1910]. They found that whereas, when exposed to the action of bromine for 24 hours at 21° only 5·10 % of *d*-galactose remained and 22·20 % of *d*-glucose, yet there was 100·00 % of *d*-fructose.

Action of dilute acid (N/8) on fructose.

On considering the results shown in Table I, it was thought that it might be of interest to determine what percentage of fructose apparently lost by oxidation in these experiments might have been really destroyed by the action of dilute acid on this sugar.

Accordingly to test this the following experiments were carried out, the procedure being exactly the same as in the standard experiments (Table I) except that no bromine was added. A certain portion of the sugar solution made by dissolving a weighed quantity of fructose in water was taken and the amount of sugar present determined, in one case by reduction by Kendall's method and in the other by polarimetric observations. Then 50 cc. of the original solution were diluted to 70 cc. and 10 cc. normal sulphuric acid added giving a solution of $\frac{1}{8}$ normal acidity which is the original strength of the acid in the oxidation experiments. This solution having been shaken to mix was allowed to stand for 66 hours after which period it was made up to a known volume, then an aliquot portion removed and the remaining fructose estimated by reduction. The percentage of fructose destroyed by N/8 acid under the above conditions was thus determined. It must be observed however that in experiments where a mixture is being quantitatively oxidised the acidity is greater than $\frac{1}{8}$ normal owing to the production of acid in the usual way when halogen oxidation occurs and moreover owing to aldonic acids produced from the sugars.

The results of these experiments are shown in Table IX where the figures represent the weight of fructose in grams.

TABLE IX.

	Taken	Found	Left	Percentage destroyed
A.	0·4953	0·4715	0·4664	1·061
B.	0·6740	(0·647)	0·6688	0·771

The sugars used in the above experiments were: in (A) Kahlbaum's pure "laevulose cryst" and in (B) Kahlbaum's "laevulose from Inulin II," both having been dried by exposure in finely powdered layers to sulphuric acid in a vacuum desiccator for several weeks. It will be noted that in experiment (A) the percentage destruction is calculated from the fructose found, not from the sugar weighed, since the "laevulose cryst" is not so pure as "laevulose from Inulin II," and so the value for actual fructose taken as found by reduction is used, whereas in experiment (B) where the sugar

was estimated by polarimetric readings, and the instrument available only read accurately to one-tenth of a degree, the percentage destruction is calculated from the actual amount of sugar weighed out.

It will also be apparent that in the case where the "laevulose cryst" is used, i.e. the less pure of the two qualities of sugar used (laevulose from Inulin II being when dried really an extremely pure sample), the loss indicated is greater than in the second case.

Summary.

1. Glucose and maltose are quantitatively oxidised by bromine at room temperature when allowed to stand in a saturated solution. However the liquid still retains a small amount of its original reducing power towards alkaline copper solutions.

2. Fructose is not oxidised at all, or only to a very small extent, by bromine under these conditions.

3. In the presence of deci-normal sulphuric acid there is a slight loss of fructose in 66 hours at room temperature.

4. A mixture of sucrose, maltose, glucose and fructose may be analysed as follows:

(*a*) The amount of sucrose is determined by polarisation and reduction before and after treatment with invertase.

(*b*) The resulting mixture of reducing sugars is treated with bromine under standard conditions of acidity and temperature for a given time. The aldehydic sugars, glucose (including the portion derived from sucrose) and maltose are oxidised. The ketonic sugar, fructose (including the portion derived from sucrose), remains behind. The reducing power of the solution may then be taken as due to the fructose only.

(*c*) The rotations due to the fructose and sucrose are then allowed for in the initially determined rotation. This gives the rotation due to glucose and maltose.

(*d*) The reduction due to fructose is subtracted from the initial reduction. This gives the reduction due to glucose and maltose. From these data two equations can be constructed which when solved by the usual algebraical method give the amounts of the two remaining sugars. When only glucose and fructose are present the bromine method enables the latter to be directly determined, instead of indirectly by solving an equation.

5. The addition of dilute alkali to a solution of glucose, fructose or

maltose produces a large temporary increase in its reducing power. Consequently in analytical work sugar solutions must be kept neutral or very faintly acid.

6. From a study of the electrical conductivity of a mixture of sodium hydroxide and glucose some indication has been obtained of the formation of a compound between these two substances.

7. An unsuccessful attempt was made to modify Barfoed's solution for quantitative work, by reducing the reaction to the first order instead of the fifth.

The expenses of this research were defrayed by the funds of the School of Botany, Trinity College, Dublin and we are indebted to Prof. H. H. Dixon for facilities for carrying on the work. Our thanks are also due to Prof. S. Young for the use of apparatus in the Chemical Laboratory.

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